TNO-Report for the study entitled:

Effect of increasing amounts of dietary fat on plasma concentrations of cholesterol, PFOS, and PFOA plasma in male APOE*3-Leiden.CETP mice fed approximately 0.03 ppm of PFOS and PFOA

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Summary

Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are exceptionally stable, amphiphilic, anionic compounds with strong surface-active properties and are essentially completely ionized in nature. Both PFOA and PFOS are widely distributed in nature and are present in the blood (serum/plasma) of the human general population at low ng/mL concentrations. Once present in the body of humans, both compounds clear slowly, with estimated serum elimination half-lives of several years. Although both PFOA and PFOS have been found to reduce serum cholesterol in experimental studies, epidemiological studies have found positive associations of both PFOA and PFOS with serum non-HDL cholesterol at serum PFOA and PFOS concentrations below approximately 50 ng/mL. At greater serum PFOA and PFOS concentrations, these associations are either much weaker or not present. The paradoxical associations found at low serum concentrations have prompted the investigation of potential non-causal factors that may account for positive associations between serum cholesterol and general population serum concentrations of PFOA and PFOS.

One potential non-causal hypothesis for the observed associations is that dietary factors that may affect serum cholesterol may also affect serum PFOA and PFOS in the same direction. Based on dietary questionnaire data, serum concentrations of PFOA and PFOS among pregnant Danish women were found to be positively associated with higher consumption of dietary fat, among other factors. However, in another study based on the United States Centers for Disease Control National Health and Nutrition Examination Survey, questionnaire-based consumption of dietary saturated fat was not associated with serum PFOA and PFOS.

Epidemiological and observational studies have noted that intake of saturated fat is associated with increased plasma lipid levels. The experiment reported herein was based on the hypothesis that, assuming equal oral intake of PFOS or PFOA on a mass/kg body-weight basis, absorption of PFOS or PFOA from the gastrointestinal tract will increase with increasing dietary fat in a similar manner to cholesterol. Based on increased absorption, plasma concentrations of cholesterol, PFOS, and PFOA will also increase with increasing dietary fat consumption.

Aim: to test the hypothesis that increased consumption of saturated fat leads to both increased plasma lipid and increased plasma PFOS and PFOA concentrations, using male E3L.CETP mice on diets with increasing amounts of saturated fat with equal dietary doses of PFOS and PFOA. The PFOS and PFOA doses used do not affect plasma lipid levels and result in PFOS and PFOA plasma levels in the range of those found in the general population (determined from previous studies of the sponsor).

Forty male APOE*3Leiden.CETP mice on a regular chow diet were weighed and blood samples were taken after a 4-hour fasting period to measure plasma cholesterol and triglycerides (t=-4 weeks). Thereafter, the mice were sub-divided into four groups of 10 mice each, matched for body weight, plasma total cholesterol and triglycerides, and the 4 weeks run-in period was started. Group 1 received a diet with 5% fat, group 2 received a diet with 15% fat, group 3 received a diet with 25% fat, and group 4 received a diet with 35% fat. At t=-3 and t=-2 weeks food intake was measured. At t=0 weeks body weight and food intake were measured and blood samples were taken after a 4-hour fasting period to measure plasma total cholesterol, HDL-cholesterol and triglycerides. The run-in period was prolonged with one week, hereafter the 4 weeks treatment period was started. Mice were treated with the different diets with PFOA/PFOS (nominal dose 0.03 ppm for the diet with 35% fat and equal oral amounts for the other diets based on food intake data of t=-3 weeks and -2 weeks). Food intake was measured weekly during treatment. At t=2 and 4 weeks, body weight was measured and blood samples were taken after a 4-hour fasting period to measure plasma cholesterol, HDL-cholesterol and triglycerides. At t=-4, 0, 2 and 4 weeks lipoprotein profiles were measured at group level. Extra plasma samples were collected at t=-4, 0, 1, 2 and 4 weeks for PFOA/PFOS analysis. Mice were sacrificed, and plasma was collected via heart puncture and liver was isolated and weighed.

Markers of general safety and well-being

- Specific clinical signs were not observed during the study. At sacrifice, macroscopic aberrations were not observed.

Body weight, liver weight and food intake

- The different fat percentages in the diets did not significantly influence body weights. Only at t=2 weeks, body weights were significantly higher in the 15% fat + PFOA/PFOS treatment when compared to the 25% fat + PFOA/PFOS treatment.
- Liver weights were decreased by the fat concentration in the different diets in a fat-concentration-dependent manner. When compared to the 5% fat diet + PFOA/PFOS group, the largest reduction was seen in the 35% fat + PFOA/PFOS group (by -24%, p=0.001).
- The different fat diets did not significantly influence food intake, except for t=-2 weeks and at t=2 weeks. At t=-2 weeks, the group on 35% fat diet showed a significantly lower food intake when compared to the group on 5% fat diet (by -18%, p=0.029); at t=2 weeks, the group on 25% fat diet + PFOA/PFOS showed a significantly decreased food intake when compared to the group on 15% fat diet + PFOA/PFOS (by -16%, p=0.032), consistent with the body-weight observations in these groups at t=2 weeks.

PFOA/PFOS intake and plasma and liver levels

- The PFOA and PFOS doses in the regular chow diet (mice were fed this diet until the start of the run-in period at t=-4 weeks) and in the different high fat diets without PFOA/PFOS added (mice were fed these diets during the run-in period), were below the lower limit of quantitation (LLOQ, 0.5 ng/g). At t=1, 2, and 4 weeks, PFOA plasma levels were decreased in a dietary fat-concentration-dependent manner. PFOS plasma levels were decreased by the 15%, 25% and 35% fat diets at t=2 and 4 weeks, when compared to the 5% fat diet levels. The PFOA and the PFOS intakes were significantly different between most of the groups at the different time points of the treatment period. Therefore, the measured plasma PFOA/PFOS levels were corrected for PFOA and PFOS intake, by calculating the estimated PFOA/PFOS burden in the body as % of PFOA/PFOS intake.
- When corrected for the PFOA and PFOS intake, the estimated PFOA and PFOS burden in the body at the different time points were decreased by the different fat diets in a fat-concentration-dependent manner. When comparing the 5% fat diet group the group to the 35% fat diet, the 35% fat diet showed the highest reduction in estimated whole-body PFOA content, estimated based on plasma PFOA concentration and volume of distribution (200 mL/kg) at all time points (by -29% (p=0.002), -27% (p=0.001), and -27% (p=0.009) for 1, 2, and 4 weeks, respectively). When comparing to the 5% fat diet group to the 35% fat diet group, the 35% fat diet group also had significantly lowered estimated whole-body PFOS content at all time points (by -47% (p=0.001), -55% (p<0.001) and -50% (p<0.001) after 1, 2, and 4 weeks of treatment, respectively).
- Both total liver PFOS and PFOA levels (liver PFOS or PFOA concentration times liver weight) seemed to be decreased by the different fat diets in a fat-concentration dependent manner. Liver PFOA and PFOS levels as percentage of PFOA/PFOS intake, seemed to be decreased by the different fat diets in a fat-concentration dependent manner. When compared to the 5% fat diet group the group on 35% fat diet showed a significant reduction in liver PFOA and PFOS (by -26% (p=0.019) and by -61% (p=0.003); respectively).

Plasma lipids

- Prior to the run-in period when mice were on a regular chow diet, plasma total cholesterol levels were similar in all groups (approximately 2.5 mM). The fat content in the semi-synthetic diets had a major effect on plasma total cholesterol levels in a dietary fat-concentration-dependent manner. Plasma total cholesterol ranged from approximately 12 mM for the 5% fat diet to approximately 5 mM for the 35% fat diet. When comparing the lowest fat content diet (5% fat) with the highest (35% fat), the latter showed a robust reduction of total cholesterol levels (by -59% (p<0.001), -65% (p<0.001), and -61% (p<0.001) at t=0, 2, and 4 weeks, respectively).
- Prior to the run-in period when mice were on a regular chow diet, plasma triglycerides levels were similar in all groups. The fat content in the diets had a major effect on plasma triglycerides levels in a dietary fat-concentration-dependent manner and in a similar direction as plasma total cholesterol levels were affected. When comparing the lowest fat content diet (5% fat) with the highest (35% fat), the latter showed a robust reduction of triglycerides levels (by -51% (p=0.001), -61% (p<0.001), and -54% (p<0.001) at t=0, 2, and 4 weeks, respectively).
- The fat content in the diets had a major effect on plasma HDL-cholesterol levels in a dietary fat-concentration-dependent manner. When comparing the lowest fat content diet (5% fat) with the highest (35% fat), the latter showed a robust elevation of HDL-

cholesterol levels (by +147% (p=0.001), +171% (p<0.001) and +140% (p<0.001) at t=0, 2, and 4 weeks, respectively).

- The lipoprotein profiles were in line with the measured plasma lipid levels. At t=-4 weeks (mice were on a regular chow diet), profiles were similar between groups. Fat in the diets decreased the ApoB-containing lipoproteins (VLDL and LDL) and increased HDL in a fat-concentration dependent manner.

Conclusion

In male ApoE3L.CETP mice, the increased consumption of saturated fat led to decreased plasma total cholesterol and triglycerides levels and increased HDL-cholesterol levels. Because intake of PFOA and PFOS was not equal between mice on the different fat diets, uptake of PFOA and PFOS from the diets was estimated based on plasma PFOA and PFOS concentrations and volume of distribution compared against PFOA and PFOS intake based on diet consumption and dietary concentrations of PFOA and PFOS. The corrected estimated uptake of PFOA and PFOS levels were decreased with increasing consumption of saturated fat. Liver PFOA and PFOS levels, measured at the end of the study, confirmed the plasma results; decreased PFOA and PFOS levels with increasing consumption of saturated fat. The study indicated that in male ApoE3L.CETP mice, the increased consumption of saturated fat led to decreased PFOA and PFOS uptake and plasma concentrations while at the same time leading to decreased plasma total and non-HDL cholesterol. The results of the current study in mice do not support the hypothesis that increasing dietary fat increases the uptake and plasma concentrations of PFOA and PFOS while also increasing the plasma concentration of cholesterol.

Although the original hypothesis was not demonstrated, an important finding emerges from the experiment. Increasing dietary fat consumption in the presence of low and constant dietary concentrations of PFOA and PFOS leads not only to lower serum non-HDL cholesterol, but also lower serum PFOA and PFOS plasma concentrations, resulting from lower uptake of PFOA and PFOS. Therefore, plasma PFOA and PFOS were associated with plasma total and non-HDL cholesterol, a finding similar to that observed in epidemiological studies. The basis of these associations is not known currently, and the extent to which these experimental findings may explain the epidemiological associations observed in human populations is also not known. However, this experiment does demonstrate that a dietary factor, increasing fat, can create associations in ApoE3L.CETP mice similar to those observed in human populations in the same range of serum PFOA and PFOS concentrations.

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1. Introduction & aim

Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are exceptionally stable, amphiphilic, anionic compounds with strong surface-active properties and are essentially completely ionized in nature. Both PFOA and PFOS are widely distributed in nature and are present in the blood (serum/plasma) of the human general population at low ng/mL concentrations. Once present in the body of humans, both compounds clear slowly, with estimated serum elimination half-lives of several years. Although both PFOA and PFOS have been found to reduce serum cholesterol in experimental studies, epidemiological studies have found positive associations of both PFOA and PFOS with serum non-HDL cholesterol at serum PFOA and PFOS concentrations below approximately 50 ng/mL. At greater serum PFOA and PFOS concentrations, these associations are either much weaker or not present. The paradoxical associations found at low serum concentrations have prompted the investigation of potential non-causal factors that may account for positive associations between serum cholesterol and general population serum concentrations of PFOA and PFOS.

One potential non-causal hypothesis for the observed associations is that dietary factors that may affect serum cholesterol may also affect serum PFOA and PFOS in the same direction. Based on dietary questionnaire data, serum concentrations of PFOA and PFOS among pregnant Danish women were found to be positively associated with higher consumption of dietary fat, among other factors. However, in another study based on the United States Centers for Disease Control National Health and Nutrition Examination Survey, questionnaire-based consumption of dietary saturated fat was not associated with serum PFOA and PFOS.

Epidemiological and observational studies have noted that intake of saturated fat is associated with increased plasma lipid levels. The experiment reported herein was based on the hypothesis that, assuming equal oral intake of PFOS or PFOA on a mass/kg body-weight basis, absorption of PFOS or PFOA from the gastrointestinal tract will increase with increasing dietary fat in a similar manner to cholesterol. Based on increased absorption, plasma concentrations of cholesterol, PFOS, and PFOA will also increase with increasing dietary fat consumption.

Aim: to test the hypothesis that increased consumption of saturated fat leads to both increased plasma lipid and increased plasma PFOS and PFOA concentrations, using male E3L.CETP mice on diets with increasing amounts of saturated fat with equal dietary doses of PFOS and PFOA. The PFOS and PFOA doses used do not affect plasma lipid levels and result in PFOS and PFOA plasma levels in the range of those found in the general population (determined from previous studies of the sponsor).

2. Materials and methods

2.1 Test substance and reference substance

Test substances: Perfluorooctanoic acid, ammonium salt, NH_4^+PFOA, lot 15822
Perfluorooctanesulfonate, potassium salt, K^+PFOS: FC-95, lot 217

Both substances were supplied by the 3M company (PFOS was sent to TNO on 10-Jun-04, PFOA was sent to TNO on 19-Aug-09). Upon receipt, the substances were stored at 4°C.

2.2 Mice

40 Male APOE*3Leiden.CETP transgenic mice, (10-11 weeks of age at the start of the run-in period), from the SPF breeding stock at TNO-Biosciences (Leiden) were used, and were housed during the experiment in macrolon cages (1-4 mice per cage), in clean-conventional animal rooms at TNO-Leiden (relative humidity 50-60%, temperature ~21°C, light cycle 7 am to 7 pm). Individual animals were marked by ear punch-holes. Mice were supplied with food and acidified tap water *ad libitum.*

2.3 Animal welfare

Experiments were performed in conformance with the rules and regulations set forward by the Netherlands Law on Animal Experiments. Experiments had been approved by the Animal Experiment Committee of TNO under registration number 2956. The study director was entitled to terminate experiments in case of serious unexpected animal discomfort.

2.4 Diets

Semi-synthetic diets were used that contained different amounts of cacao butter and sucrose and equal amounts of corn oil, cholesterol and casein based on an isocaloric intake. Sucrose in the diets was replaced by cacao butter to a fat concentration in the diet of 5%, 15%, 25 % and 35%. All diets were prepared by ABdiets (Woerden, Netherlands).

Description	kCal per gram	5% fat diet		15% fat diet		25% fat diet		35% fat diet	
		gram	kCal	gram	kCal	gram	kCal	gram	kCal
st. vit. premix	4 kCal/g	0.25	1	0.25	1	0.25	1	0.25	1
st. spor. premix	-	0.25	0	0.25	0	0.25	0	0.25	0
CaHPO4*2H2O	-	1.3	0	1.3	0	1.3	0	1.3	0
CaCO3	-	1	0	1	0	1	0	1	0
KH2PO4	-	0.7	0	0.7	0	0.7	0	0.7	0
KCl	-	0.7	0	0.7	0	0.7	0	0.7	0
NaCl	-	0.3	0	0.3	0	0.3	0	0.3	0
MgSO4*7H2O	-	0.4	0	0.4	0	0.4	0	0.4	0
MgO	-	0.2	0	0.2	0	0.2	0	0.2	0
Methionine DL	4 kCal/g	0.2	0.8	0.2	0.8	0.2	0.8	0.2	0.8
Choline CL 50%	-	2	0	2	0	2	0	2	0
acid casein	3.61 kCal/g	20	72.2	20	72.2	20	72.2	20	72.2
cellulose/diacel	-	6.2	0	6.2	0	6.2	0	6.2	0
corn starch	3.664 kCal/g	10	36.6	6.9	25.3	4.4	16.1	2.3	8.4
sucrose	4 kCal/g	51.5	206	35.5	142	22.6	90.4	12	48
Cocoa butter	9 kCal/g	4	36	12.4	111.6	19.2	172.8	24.7	222.3
corn oil	9 kCal/g	1	9	1	9	1	9	1	9
Cholesterol	-	0.223	0	0.223	0	0.223	0	0.223	0

	gram	kcal	gram	kcal	gram	kcal	gram	kcal
total	100	362	90	362	81	362	74	362
% fat	5%		15%		25%		35%	
kcal/gram		3.61		4.04		4.48		4.91

2.5 Drug administration

Equal amounts of PFOA and PFOS were administered orally as admix to the 4 different diets for 4 weeks (0.03 ppm for the diet with 35% fat and equal oral amounts for the other diets based on food intake data of t=-3 weeks and -2 weeks). Blood collections at the start of the study (t=-4 weeks) and following the run-in period (t=0 weeks) were added to be able to measure the background levels of PFOS and PFOA in plasma of mice on chow diet and on the different diets without added PFOS and PFOA. To be able to measure the accumulation of PFOS and PFOA in

plasma during treatment, blood was collected after 1, 2 and 4 weeks of treatment. Every week diets were changed and left over diet was collected per cage.

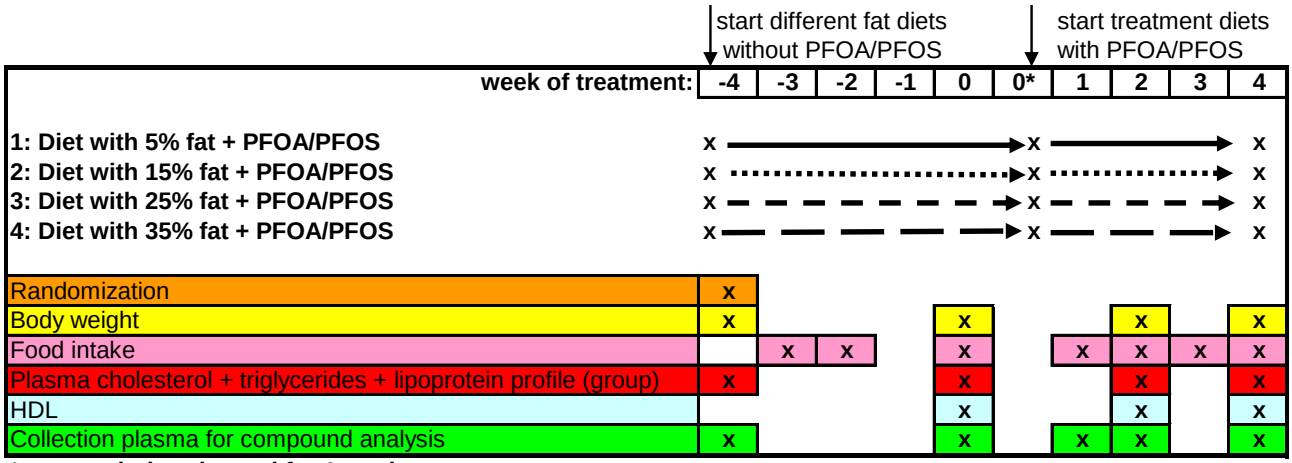
2.6 Treatment groups

1) Diet with 5% fat + PFOS/PFOA
2) Diet with 15% fat + PFOS/PFOA
3) Diet with 25% fat + PFOS/PFOA
4) Diet with 35% fat + PFOS/PFOA

2.7 Study design

The study was performed according to the scheme as outlined in figure 1.

Figure 1. Flow chart of the study



***: run-period prolonged for 1 week**

Forty male APOE*3Leiden.CETP mice on a regular chow diet were weighed and blood samples were taken after a 4-hour fasting period to measure plasma cholesterol and triglycerides (t=-4 weeks). Thereafter, the mice were sub-divided into four groups of 10 mice each, matched for body weight, plasma total cholesterol and triglycerides, and the 4 weeks run-in period was started. Group 1 received a diet with 5% fat, group 2 received a diet with 15% fat, group 3 received a diet with 25% fat, and group 4 received a diet with 35% fat. At t=-3 and t=-2 weeks food intake was measured. At t=0 weeks body weight and food intake were measured and blood samples were taken after a 4-hour fasting period to measure plasma total cholesterol, HDL-cholesterol and triglycerides. The run-in period was prolonged with one week, hereafter the 4 weeks treatment period was started. Mice were treated with the different diets with PFOA/PFOS (nominal dose 0.03 ppm for the diet with 35% fat and equal oral amounts for the other diets based on food intake data of t=-3 weeks and -2 weeks). Food intake was measured weekly during treatment. At t=2 and 4 weeks, body weight was measured and blood samples were taken after a 4-hour fasting period to measure plasma cholesterol, HDL-cholesterol and triglycerides. At t=-4, 0, 2 and 4 weeks lipoprotein profiles were measured at group level. Extra plasma samples were collected at t=-4, 0, 1, 2 and 4 weeks for PFOA/PFOS analysis. Mice were sacrificed, and plasma was collected via heart puncture and liver was isolated and weighed.

One day after the last blood sampling, mice (non fasted) were sacrificed and plasma was collected via heart puncture and liver was isolated and weighed. Two pieces of liver tissue were snap-frozen in liquid nitrogen for optional measurements, one piece of liver was fixed in 10% formalin for optional histology analysis.

The in-life mouse work started on July 1st 2010 and ended on September 3rd 2010.

2.8 Determinations (see also, Figure 1)

- Body weight at week -4, 0, 2, 4.
- Food intake per cage at week -3, -2, 0, 1, 2, 3, 4.
- Plasma cholesterol and triglycerides after 4h fasting at week -4, 0, 2 and 4.
- Lipoprotein profiles (cholesterol and phospholipids) at group level at week 0 and 4.
- HDL-cholesterol measurement at week 0 ,2 and 4.
- Extra plasma collection for PFOA/PFOS analysis at week -4, 0, 1, 2, 4
- Sacrifice: heart puncture with EDTA-plasma collection, liver weight, collection of 4 liver parts (N_2) and 1 liver part (formalin).

2.9 Methods

- Total plasma cholesterol and triglycerides were determined using kit "Cholesterol CHOD-PAP" and kit "Triglycerides GPO-PAP" both from Roche (Mannheim, Germany).
- Measurement of pooled lipoprotein profiles by FPLC analysis using an AKTA apparatus. Analyses were performed in freshly obtained samples or samples stored at 4°C for one day. Cholesterol and phospholipid profiles were measured in the fractions using kit "Cholesterol CHOD-PAP" from Roche (Mannheim, Germany) and kit "Phospholipids" from Instruchemie (Delfzijl, the Netherlands), respectively.
- Plasma HDL-cholesterol levels were determined using kit "Cholesterol CHOD-PAP" from Roche (Mannheim, Germany) after the precipitation of ApoB-containing lipids by $MnCl_2$ (0.2 mol/L) and heparin (500 IU/mL).

2.10 Sacrifice

Mice were sacrificed via CO_2 suffocation. After sacrifice, blood was collected via heart puncture in CB 300 K2E microvettes (Sarstedt, Nürnbrecht, Germany) containing EDTA-dipotassium salt. Microvettes containing blood were placed on ice immediately. EDTA-plasma was obtained after centrifugation (10 min at 6000 rpm) of the samples in a lab centrifuge at 4°C and samples were then stored at -80°C. Livers were isolated and weighed and two parts were snap-frozen in liquid N_2 and stored individually at -80°C, one part was fixed in 10% buffered formalin solution.

2.11 Statistical analysis

Significance of differences between the groups were calculated non-parametrically, using the computer program SPSS. For non-parametric calculations a Kruskall-Wallis test for several independent samples was used, followed by a Mann-Whitney U-test for independent samples.

3. Deviations from the protocol

The following deviations from the original protocol were observed.

Since at t=0, the results of the plasma lipid measurements of the different groups were unexpected, start of treatment was postponed for one week to discuss the results of the t=0 weeks measurements with the Sponsor (email correspondence of 02 Aug 2010).

It was decided not to exclude 2 mice per group during the treatment period (so the number of mice per group during the treatment period remained 10).

Extra food intake measurements were performed at t=1 and t=3 weeks to be able to calculate more precisely the PFOA/PFOS intake.

After discussion with the sponsor, it was decided to also perform HDL-cholesterol measurements at t=2 weeks (see email correspondence 13-14 April 2011).

At sacrifice, EDTA-plasma was collected via heart puncture and liver was isolated and weighed. Four pieces of liver tissue were snap-frozen in liquid nitrogen for optional measurements; one piece of liver was fixed in 10% formalin for optional histology analysis. The sponsor used one of the liver pieces for PFOA and PFOS liver level measurements.

4. Results

4.1 Markers of general well-being

No adverse clinical signs were observed during the study. At sacrifice, no abnormal macroscopic aberrations were observed.

4.2 Body weight

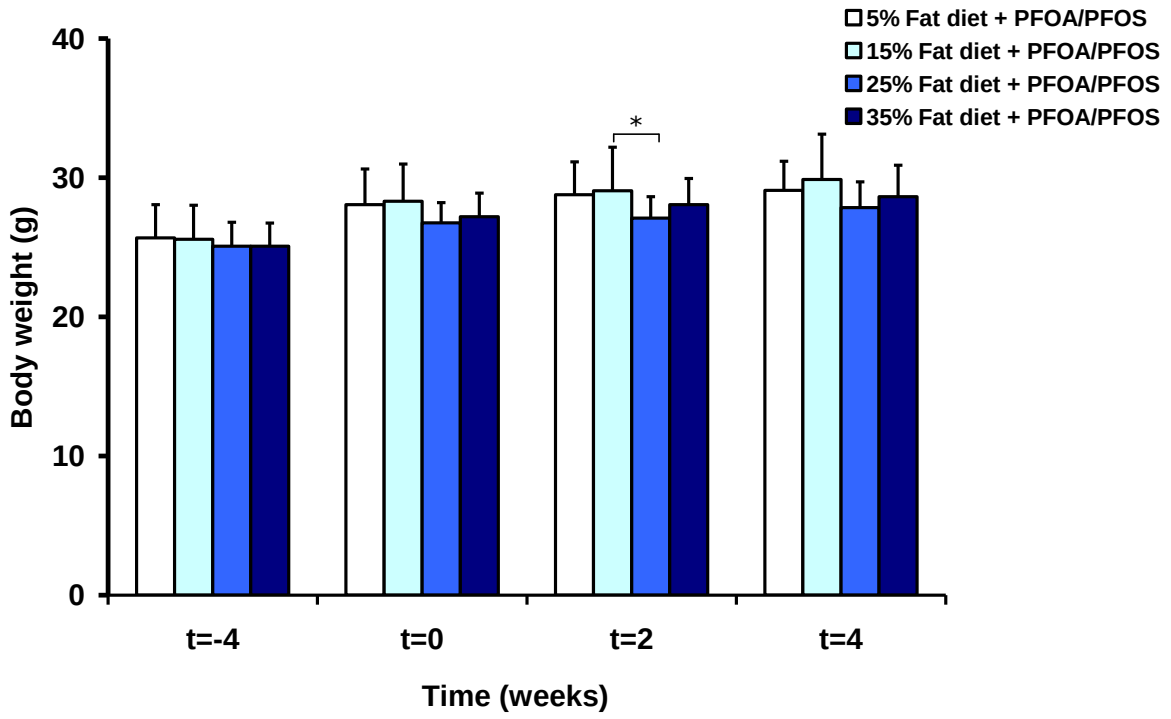
Values are absolute values (grams) and are means ± S.D. of 10 mice per group. Individual body weight per mouse and number of mice per group are given in appendix I.

Body weight (g) Time (weeks)					
Group		**t=-4**	**t=0**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	25.7	28.1	28.8	29.1
	SD	2.4	2.6	2.4	2.1
15% fat + PFOA/PFOS	AVG	25.6	28.3	29.1	29.9
	SD	2.4	2.7	3.1	3.3
25% fat + PFOA/PFOS	AVG	25.1	26.8	27.1	27.9
	SD	1.7	1.5	1.5	1.8
35% fat + PFOA/PFOS	AVG	25.1	27.2	28.1	28.6
	SD	1.7	1.7	1.9	2.3

Body weight, P value		Time (weeks)			
		t=-4	**t=0**	**t=2**	**t=4**
Between groups		0.602	0.183	0.154	0.234
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.739	0.353	0.353	0.353
	25% fat + PFOA/PFOS	0.315	0.280	0.105	0.247
	35% fat + PFOA/PFOS	0.353	0.393	0.436	0.579
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.436	0.052	**0.043**	0.052
	35% fat + PFOA/PFOS	0.353	0.105	0.280	0.247
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.853	0.971	0.353	0.529

The different fat percentages in the diets did not significantly influence body weights. Only at t=2 weeks, body weights were significantly higher in the 15% fat + PFOA/PFOS treatment when compared to the 25% fat + PFOA/PFOS treatment. Since there are no significant differences between the body weights of mice fed the more extreme diets (5% and 35% fat diets), the significant difference in body weight between 15% fat + PFOA/PFOS treatment and 25% fat + PFOA/PFOS treatment is likely due to chance.

Figure 4.2 Body weight



*: p<0.05, Mann-Whitney U test

4.3 Liver weight

Values are absolute values (grams) or relative values (% of body weight) and are means ± S.D. of 10 mice per group. Individual liver weight per mouse and number of mice per group are given in appendix II.

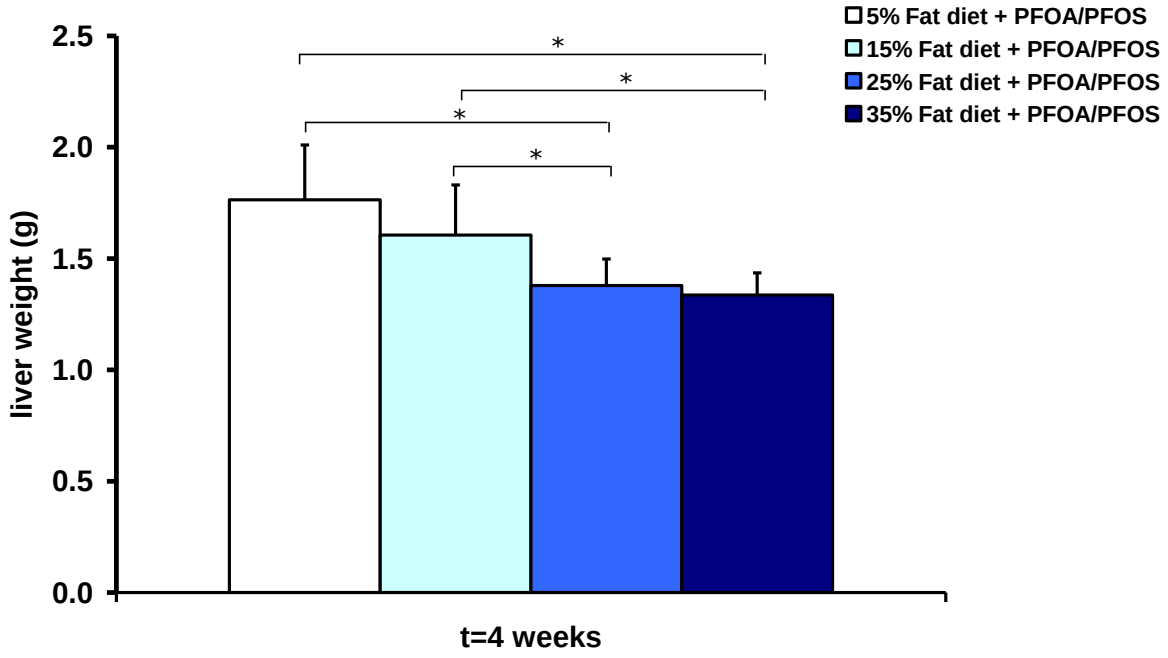
Liver weight at t=4 weeks			
Group		**g**	**% of body weight**
5% fat + PFOA/PFOS	AVG	1.8	6.1%
	SD	0.2	0.9%
15% fat + PFOA/PFOS	AVG	1.6	5.4%
	SD	0.2	0.4%
25% fat + PFOA/PFOS	AVG	1.4	5.0%
	SD	0.1	0.3%
35% fat + PFOA/PFOS	AVG	1.3	4.7%
	SD	0.1	0.2%

Liver weight, P value		**g**	**% of body weight**
Between groups		**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.089	**0.005**
	25% fat + PFOA/PFOS	**0.001**	**0.002**
	35% fat + PFOA/PFOS	**0.001**	**0.002**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	**0.015**	0.063
	35% fat + PFOA/PFOS	**0.005**	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.393	**0.015**

Liver weights were dose dependent decreased by the fat concentration in the different diets. When compared to the 5% fat diet + PFOA/PFOS group, the largest reduction was seen in the 35% fat + PFOA/PFOS group (by -24%, p=0.001). Relative liver weights (corrected for body weight) were

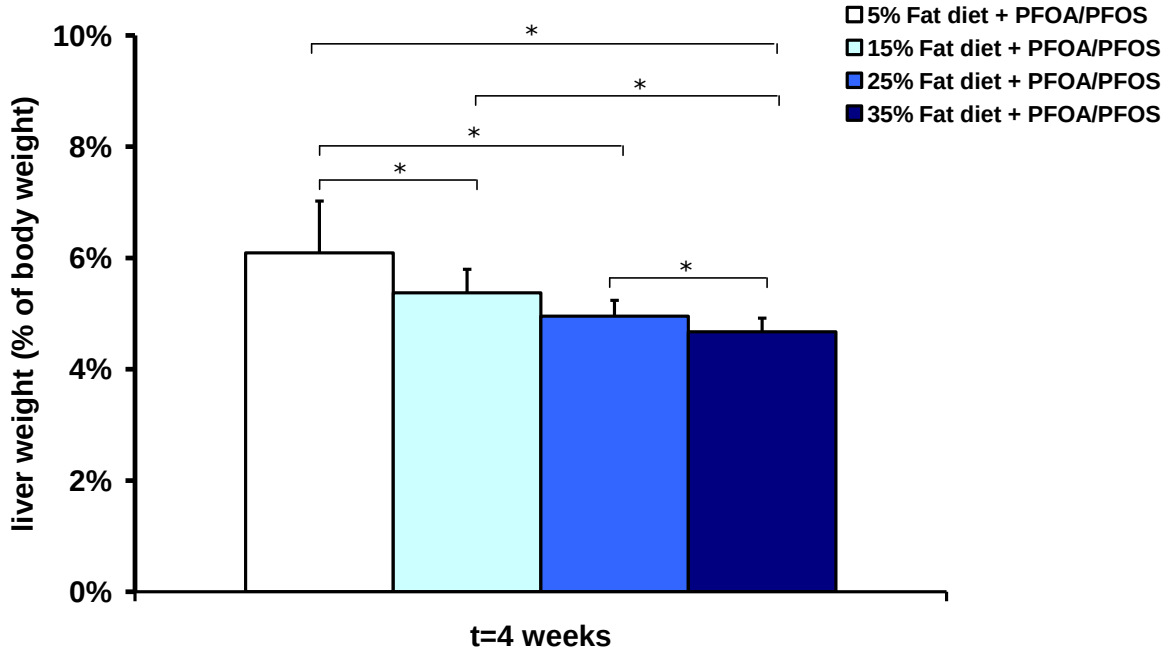
also dose dependent decreased by the fat concentration in the different diets. When compared to the 5% fat diet + PFOA/PFOS group, the largest reduction in relative liver weight was seen in the 35% fat + PFOA/PFOS group (by -23%, p=0.001).

Figure 4.3a Absolute liver weight



*: p<0.05, Mann-Whitney U test

Figure 4.3b Liver weight as % of body weight



*: p<0.05, Mann-Whitney U test

4.4 Food and fat intake

Values are absolute values (gram/mouse/day) and are means ± S.D. of 2-5 cages per group. Individual food and fat intake data per cage and number of cages per group are given in appendix III.

Food intake (g/day/mouse) Time (weeks)								
Group		**t=-3**	**t=-2**	**t=0**	**t=1**	**t=2**	**t=3**	**t=4**
5% fat + PFOA/PFOS	AVG	3.6	3.6	3.5	3.5	3.5	3.5	3.4
	SD	0.5	0.3	0.4	0.6	0.6	0.7	0.5
15% fat + PFOA/PFOS	AVG	3.4	3.5	3.5	3.6	3.7	3.5	3.6
	SD	0.1	0.3	0.3	0.4	0.3	0.5	0.5
25% fat + PFOA/PFOS	AVG	3.1	3.3	3.1	3.1	3.1	3.2	3.2
	SD	0.2	0.5	0.3	0.3	0.2	0.3	0.3
35% fat + PFOA/PFOS	AVG	3.1	3.0	3.1	3.3	3.4	3.4	3.3
	SD	0.1	0.4	0.3	0.3	0.3	0.5	0.4

Food intake, P value		Time (weeks)						
		t=-3	**t=-2**	**t=0**	**t=1**	**t=2**	**t=3**	**t=4**
Between groups		0.120	0.074	0.152	0.277	0.191	0.811	0.628
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	no analysis*	no analysis*	1.000	0.886	0.486	1.000	0.686
	25% fat + PFOA/PFOS	no analysis*	0.343	0.413	0.286	0.286	0.556	0.730
	35% fat + PFOA/PFOS	0.057	**0.029**	0.286	0.730	1.000	0.730	0.905
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	no analysis*	no analysis*	0.063	0.111	**0.032**	0.413	0.190
	35% fat + PFOA/PFOS	no analysis*	no analysis*	0.063	0.190	0.190	0.905	0.413
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	no analysis*	0.343	0.548	0.548	0.421	0.841	0.841

*: no statistical analysis could be performed; not enough cages per group.

Fat intake (g/day/mouse) Time (weeks)								
Group		**t=-3**	**t=-2**	**t=0**	**t=1**	**t=2**	**t=3**	**t=4**
5% fat + PFOA/PFOS	AVG	0.18	0.18	0.18	0.18	0.18	0.18	0.17
	SD	0.02	0.01	0.02	0.03	0.03	0.04	0.03
15% fat + PFOA/PFOS	AVG	0.51	0.53	0.53	0.54	0.55	0.53	0.54
	SD	0.02	0.05	0.04	0.05	0.05	0.07	0.08
25% fat + PFOA/PFOS	AVG	0.78	0.83	0.79	0.78	0.78	0.79	0.80
	SD	0.05	0.12	0.07	0.07	0.05	0.07	0.07
35% fat + PFOA/PFOS	AVG	1.08	1.04	1.10	1.16	1.18	1.18	1.17
	SD	0.03	0.13	0.09	0.10	0.12	0.18	0.15

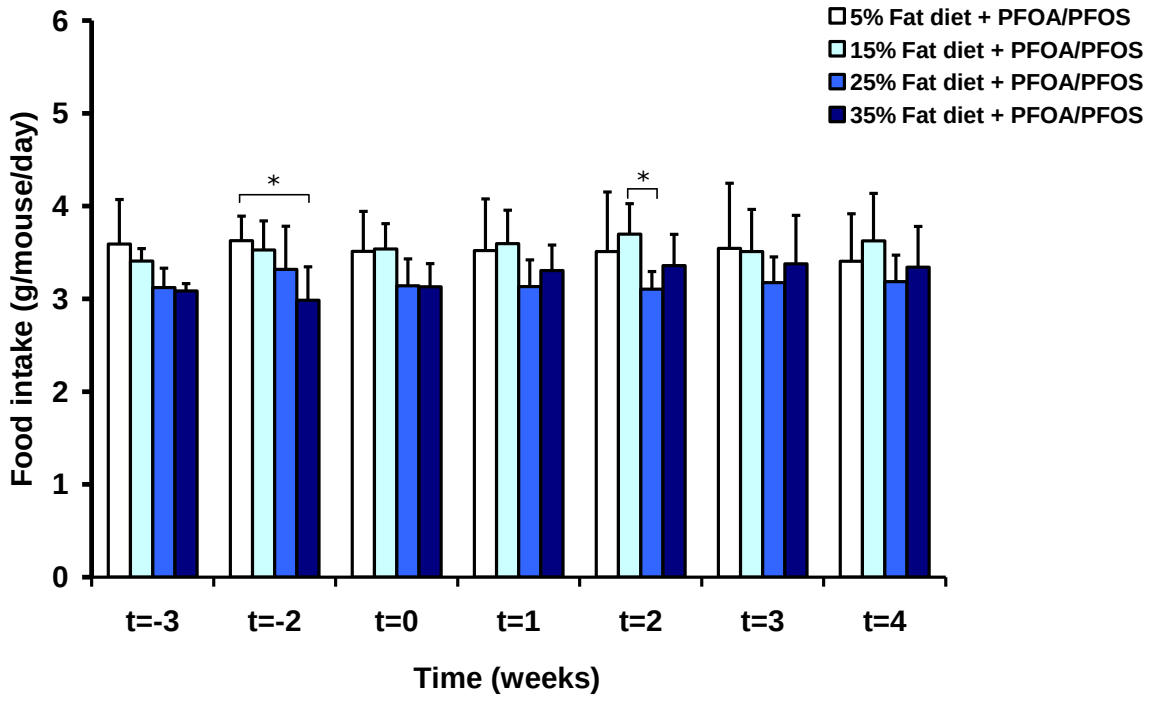
Fat intake, P value		Time (weeks)						
		t=-3	**t=-2**	**t=0**	**t=1**	**t=2**	**t=3**	**t=4**
Between groups		**0.017**	**0.005**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	no analysis*	no analysis*	**0.029**	**0.029**	**0.029**	**0.029**	**0.029**
	25% fat + PFOA/PFOS	no analysis*	**0.029**	**0.016**	**0.016**	**0.016**	**0.016**	**0.016**
	35% fat + PFOA/PFOS	**0.029**	**0.029**	**0.016**	**0.016**	**0.016**	**0.016**	**0.016**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	no analysis*	no analysis*	**0.016**	**0.016**	**0.016**	**0.016**	**0.016**
	35% fat + PFOA/PFOS	no analysis*	no analysis*	**0.016**	**0.016**	**0.016**	**0.016**	**0.016**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	no analysis*	0.057	**0.008**	**0.008**	**0.008**	**0.008**	**0.008**

*: no statistical analysis could be performed; not enough cages per group.

Although food intake seemed to be somewhat decreased by a higher fat content in the diet, the different fat diets did not significantly influence food intake, except for t=-2 weeks and at t=2 weeks. At t=-2 weeks, the group on 35% fat diet showed a significantly lower food intake when compared to the group on 5% fat diet (by -18%, p=0.029); at t=2 weeks, the group on 25% fat diet + PFOA/PFOS showed a significantly decreased food intake when compared to the group on 15% fat diet + PFOA/PFOS (by -16%, p=0.032).

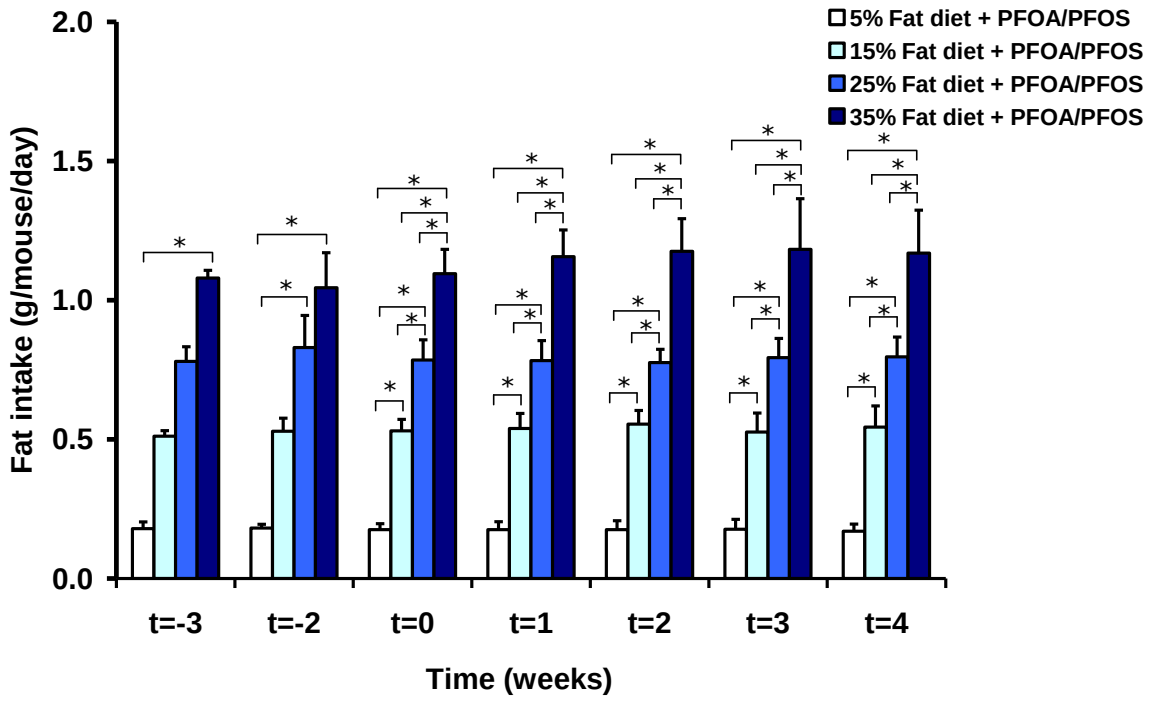
Fat intake was dose dependent increased and significantly different between all groups, except at t=-3 weeks and t=-2 weeks. At these time points not enough food intake data were available for the 15%, 25% and 35% fat groups to perform statistical analysis.

Figure 4.4a Food intake



*: p<0.05, Mann-Whitney U test

Figure 4.4b Dietary fat intake



*: p<0.05, Mann-Whitney U test

Based on the t=-3 and t=2 weeks food intake measurements the PFOA/PFOS doses in the different high fat diets were calculated. The target PFOA/PFOS doses in the different diets were 0.03 ppm PFOA/PFOS for the diet with 35% fat and equal oral intake amounts for the other diets based on food intake data of t=-3 weeks and -2 weeks. In appendix IV the calculation and measured PFOA/PFOS doses in the diets are given. The measured PFOA and PFOS doses deviated from the target PFOA/PFOS doses in all diets.

Group	**Target concentration PFOA/PFOS ng/g diet**	**Average measured concentration PFOA/PFOS in diet ng/g diet**
Group 1: 5% fat diet	36/36	28.4/17.8
Group 2: 15% fat diet	34/34	27.4/12.1
Group 3: 25% fat diet	32/32	25.3/15.5
Group 4: 35% fat diet	30/30	26.2/28.8

Based on the measured PFOA/PFOS doses in the diet and measured food intake the cumulative PFOA and PFOS dietary intake was calculated (see paragraph 4.5).

4.5 Cumulative PFOA and PFOS intake

Values are absolute values (µg/kg mouse) and are means ± S.D. of 10 mice per group. Individual PFOA and PFOS intake per mouse and number of mice per group are given in appendix IV.

Accumulated PFOA intake per kg mouse (µg/kg) Time (weeks)				
Group		**t=1**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	21.4	42.2	89.8
	SD	2.6	5.2	11.1
15% fat + PFOA/PFOS	AVG	21.1	42.1	88.2
	SD	2.4	4.4	11.2
25% fat + PFOA/PFOS	AVG	17.6	35.0	74.3
	SD	1.0	1.8	3.4
35% fat + PFOA/PFOS	AVG	18.8	37.4	79.9
	SD	1.7	3.8	9.6

Accumulated PFOA intake per kg mouse, P value		Time (weeks)		
		t=1	**t=2**	**t=4**
Between groups		0.001	0.001	0.005
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.853	0.912	0.579
	25% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
	35% fat + PFOA/PFOS	**0.035**	**0.023**	**0.043**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	**0.002**	**<0.001**	**0.019**
	35% fat + PFOA/PFOS	**0.035**	**0.035**	0.123
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.105	0.280	0.247

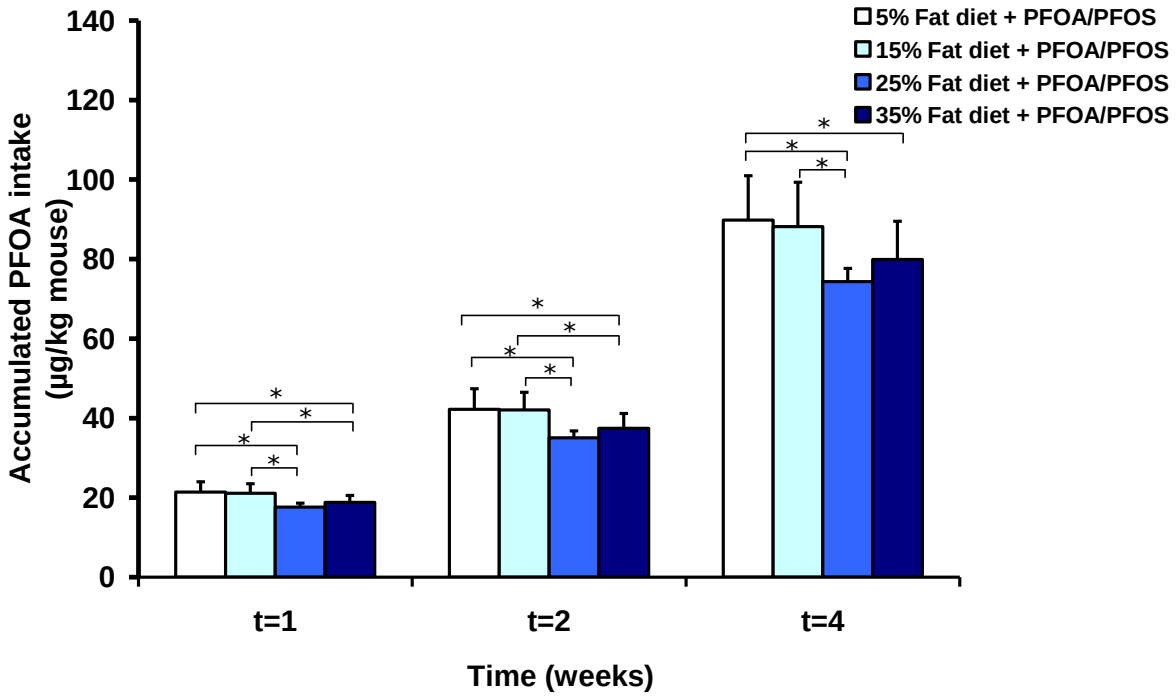
Accumulated PFOS intake per kg mouse (µg/kg) Time (weeks)				
Group		**t=1**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	13.4	26.5	56.3
	SD	1.6	3.3	7.0
15% fat + PFOA/PFOS	AVG	9.3	18.6	39.0
	SD	1.1	1.9	4.9
25% fat + PFOA/PFOS	AVG	10.8	21.4	45.4
	SD	0.6	1.1	2.1
35% fat + PFOA/PFOS	AVG	20.7	41.2	88.0
	SD	1.9	4.2	10.6

Accumulated PFOS intake per kg mouse, P value		Time (weeks)		
		t=1	**t=2**	**t=4**
Between groups		**<0.001**	**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
	25% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
	35% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	**0.002**	**0.002**	**0.001**
	35% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**

The PFOA and PFOS doses in the regular chow diet (mice were fed this diet until the start of the run-in period at t=-4 weeks) and in the different high fat diets without PFOA/PFOS added (mice were fed these diets during the run-in period), were below detection limit (<0.5 ng/mL).

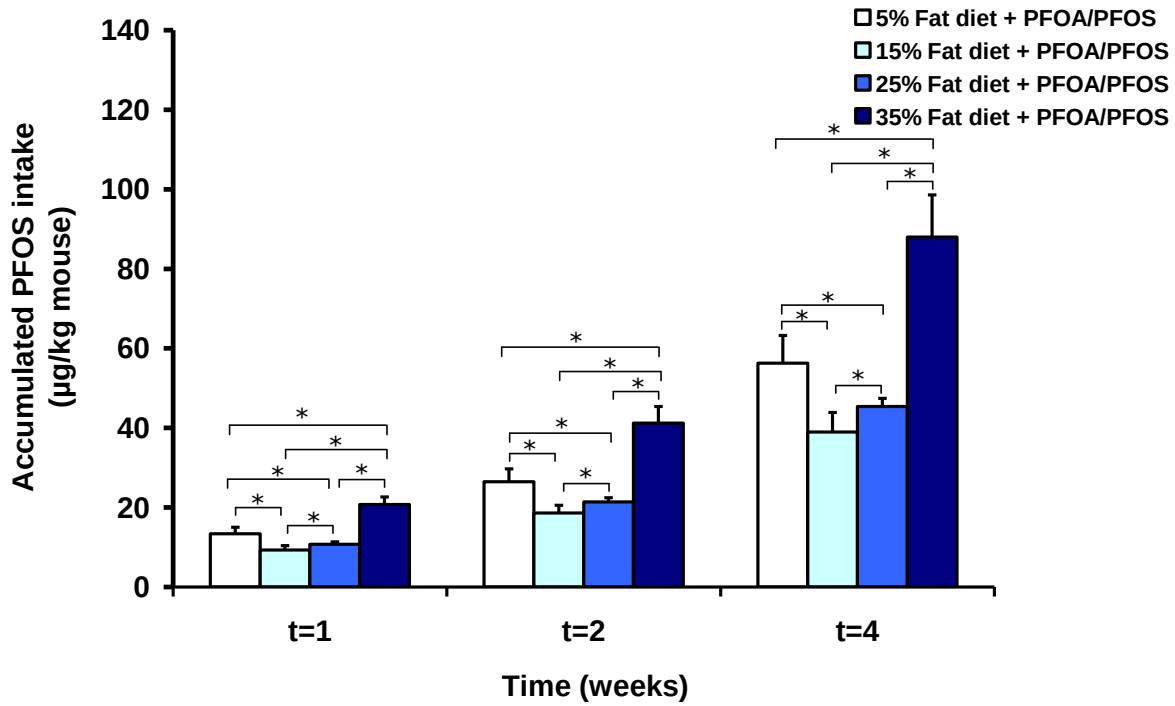
The PFOA and the PFOS intake were significantly different between most of the groups at the different time points of the treatment period. Therefore the measured plasma PFOA/PFOS levels (see paragraph 4.6) were corrected for PFOA and PFOS intake, by calculating the estimated PFOA/ PFOS dose in the body as % of PFOA/FPFOS intake (see paragraph 4.7).

Figure 4.5a Accumulated PFOA intake



*: p<0.05, Mann-Whitney U test

Figure 4.5b Accumulated PFOS intake



*: p<0.05, Mann-Whitney U test

4.6 PFOA and PFOS plasma levels

Values are absolute values (ng/mL) and are means ± S.D. of 10 mice per group. Individual PFOA and PFOS levels per mouse and number of mice per group are given in appendix V.

Plasma PFOA levels (ng/mL) Time (weeks)						
Group		**t=-4**	**t=0**	**t=1**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	<0.5	<0.6	46.0	104.0	194.7
	SD	-	-	9.7	13.4	26.2
15% fat + PFOA/PFOS	AVG	<0.5	<0.7	35.5	81.9	157.4
	SD	-	-	5.3	12.4	13.5
25% fat + PFOA/PFOS	AVG	<0.5	<0.7	29.9	65.6	143.2
	SD	-	-	2.3	5.4	13.0
35% fat + PFOA/PFOS	AVG	<0.5	<1.0	28.6	66.9	128.3
	SD	-	-	4.5	9.4	14.5

Plasma PFOA levels, P value		Time (weeks)		
		t=1	**t=2**	**t=4**
Between groups		**<0.001**	**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	**0.005**	**0.001**	**0.001**
	25% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
	35% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	**0.009**	**<0.001**	0.063
	35% fat + PFOA/PFOS	**0.015**	**0.003**	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.684	0.796	**0.023**

Plasma PFOS levels (ng/mL) Time (weeks)						
Group		**t=-4**	**t=0**	**t=1**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	<0.5	<0.5	20.5	56.9	97.5
	SD	-	-	10.8	37.1	31.8
15% fat + PFOA/PFOS	AVG	<0.5	<0.5	17.3	33.0	80.9
	SD	-	-	7.0	5.9	23.0
25% fat + PFOA/PFOS	AVG	<0.5	<0.5	15.7	27.6	69.6
	SD	-	-	6.0	6.1	27.0
35% fat + PFOA/PFOS	AVG	<0.5	<0.5	16.5	41.1	78.1
	SD	-	-	5.8	13.8	12.6

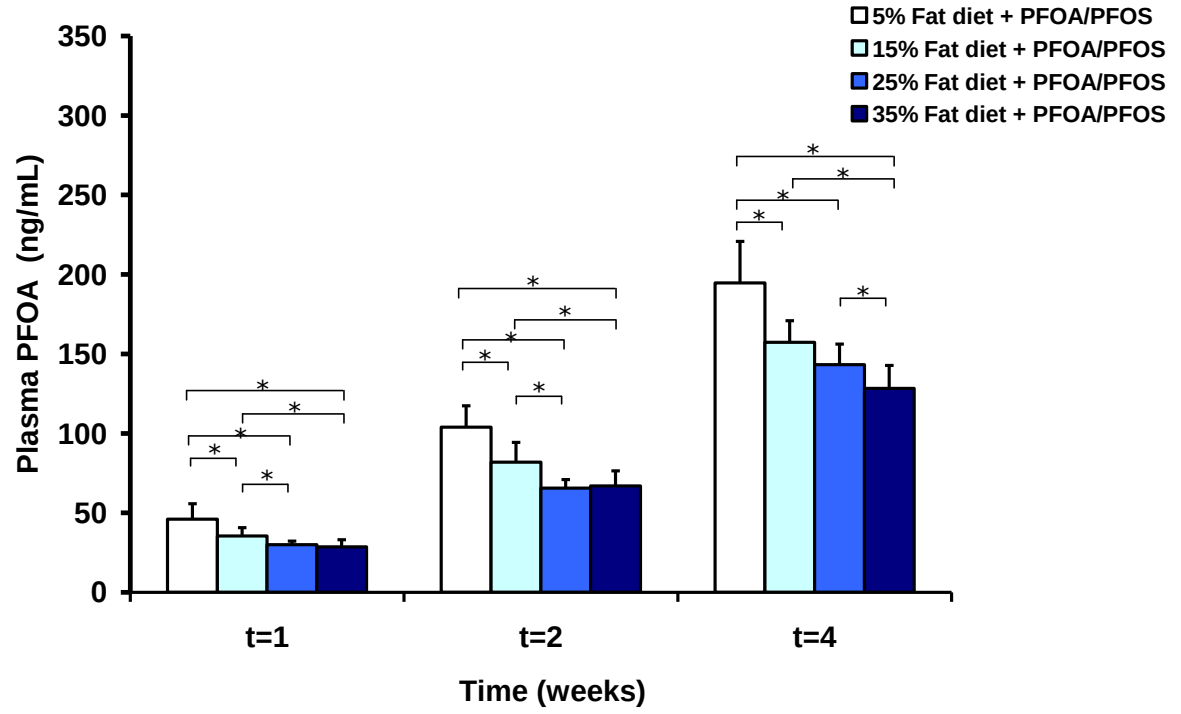
Plasma PFOS levels, P value		Time (weeks)		
		t=1	**t=2**	**t=4**
Between groups		0.564	**<0.001**	**0.010**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.280	**0.005**	0.089
	25% fat + PFOA/PFOS	0.190	**<0.001**	**0.002**
	35% fat + PFOA/PFOS	0.529	0.143	0.105
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.579	0.063	**0.043**
	35% fat + PFOA/PFOS	0.912	0.089	0.739
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.739	**0.003**	**0.043**

At the different time points, PFOA plasma levels seemed to be dose dependent decreased by the fat concentration in the different diets. Since the PFOA dietary intake was different in the different dietary fat groups, the measured plasma PFOA levels were corrected for accumulated PFOA intake, by calculating the estimated PFOA dose in the body as percentage of PFOA dietary intake (see paragraph 4.7).

When compared to the 5% fat diet, PFOS plasma levels seemed to be decreased by the 15%, 25% and 35% fat diets at t=2 and 4 weeks. Since the PFOS dietary intake was different in the different

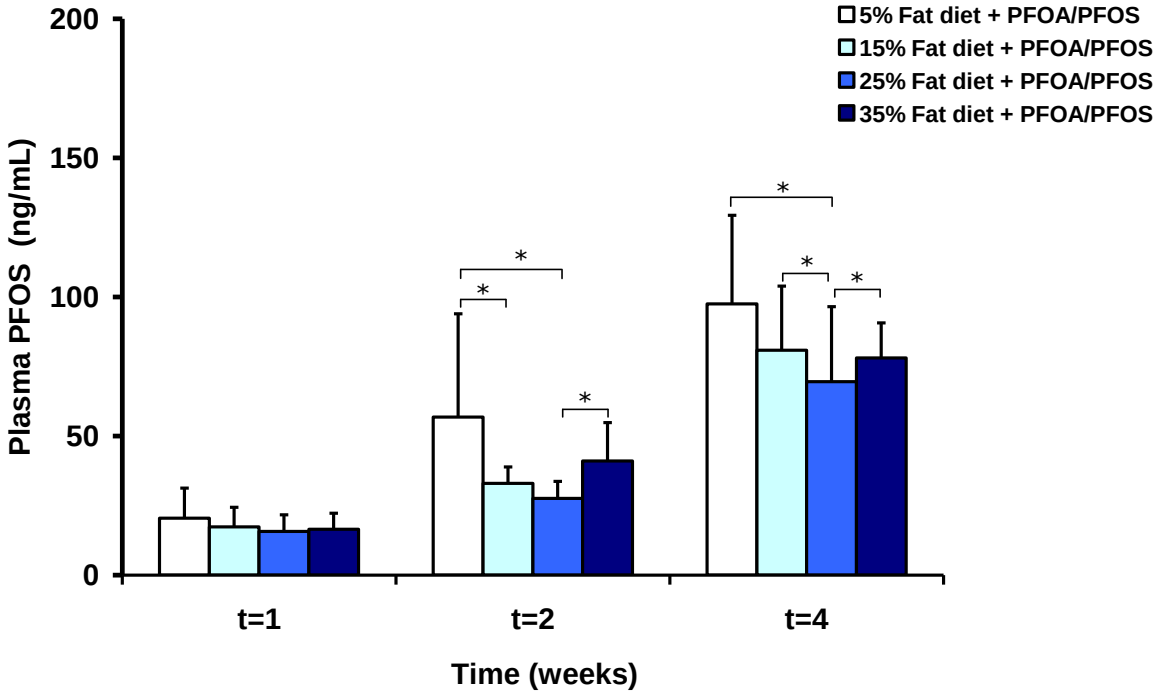
dietary fat groups, the measured plasma PFOS levels were corrected for accumulated PFOS intake, by calculating the estimated PFOS dose in the body as percentage of PFOS intake (see paragraph 4.7).

Figure 4.6a PFOA plasma levels



*: p<0.05, Mann-Whitney U test

Figure 4.6b PFOS plasma levels



*: p<0.05, Mann-Whitney U test

4.7 PFOA and PFOS dose in body as % of dietary intake

Values are relative values (%) and are means ± S.D. of 10 mice per group. Individual values per mouse and number of mice per group are given in appendix VI.

Estimated PFOA dose in body as percentage of PFOA intake (%) Time (weeks)				
Group		**t=1**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	43%	50%	44%
	SD	8%	7%	10%
15% fat + PFOA/PFOS	AVG	34%	40%	36%
	SD	8%	9%	7%
25% fat + PFOA/PFOS	AVG	34%	38%	39%
	SD	3%	3%	4%
35% fat + PFOA/PFOS	AVG	31%	36%	32%
	SD	5%	7%	4%

Estimated PFOA dose in body as percentage of PFOA intake, P value		Time (weeks)		
		t=1	**t=2**	**t=4**
Between groups		**0.008**	**0.001**	**0.018**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.052	**0.007**	0.123
	25% fat + PFOA/PFOS	**0.009**	**<0.001**	0.315
	35% fat + PFOA/PFOS	**0.002**	**0.001**	**0.009**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.631	1.000	0.393
	35% fat + PFOA/PFOS	0.353	0.315	0.280
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.063	0.247	**0.004**

Estimated PFOS dose in body as percentage of PFOS intake (%) Time (weeks)				
Group		**t=1**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	31%	44%	36%
	SD	16%	33%	15%
15% fat + PFOA/PFOS	AVG	37%	36%	41%
	SD	14%	8%	10%
25% fat + PFOA/PFOS	AVG	29%	26%	31%
	SD	10%	5%	12%
35% fat + PFOA/PFOS	AVG	16%	20%	18%
	SD	6%	6%	3%

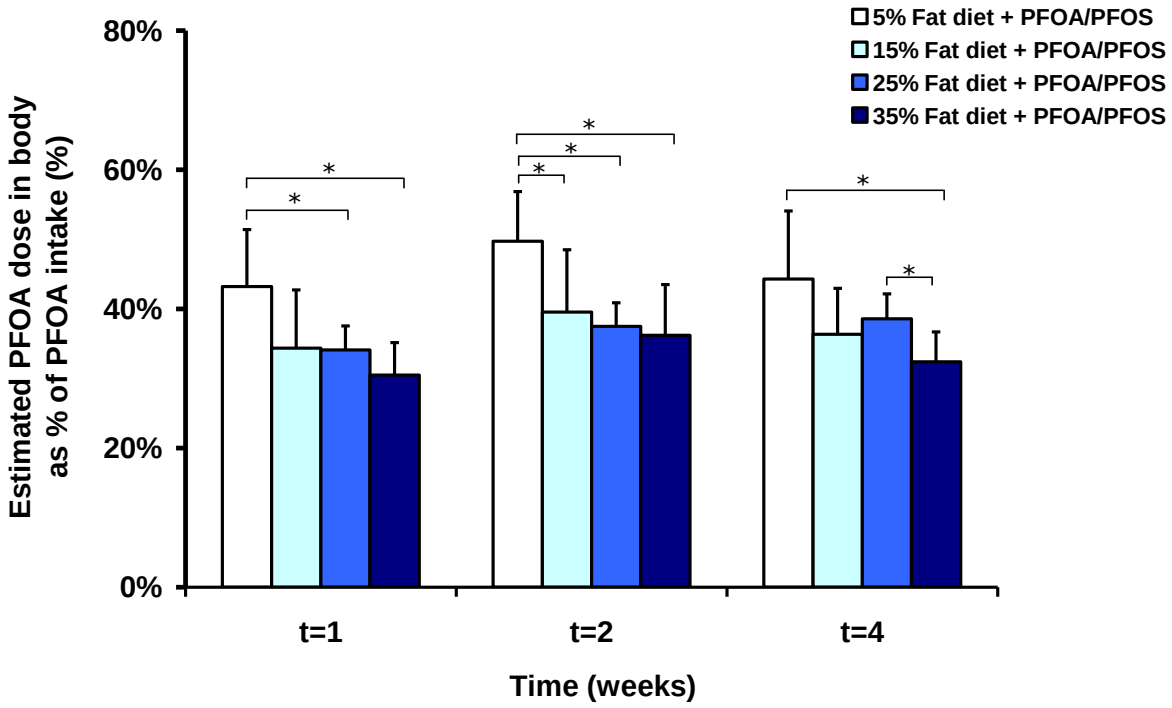
Estimated PFOS dose in body as percentage of PFOS intake, P value		Time (weeks)		
		t=1	**t=2**	**t=4**
Between groups		**<0.001**	**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.105	1.000	0.063
	25% fat + PFOA/PFOS	0.796	**0.015**	0.218
	35% fat + PFOA/PFOS	**0.001**	**<0.001**	**<0.001**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.218	**0.002**	**0.001**
	35% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	**0.002**	**0.035**	**<0.001**

When corrected for the PFOA and PFOS dietary intake, the estimated PFOA and PFOS doses in the body at the different time points seemed to be decreased by the different fat diets in a fat-concentration dependent manner.

When compared to the 5% fat diet group the group on 35% fat diet showed the highest reduction in whole body PFOA concentration at all time points (by -29% (p=0.002), -27% (p=0.001) and -27% (p=0.009); respectively).

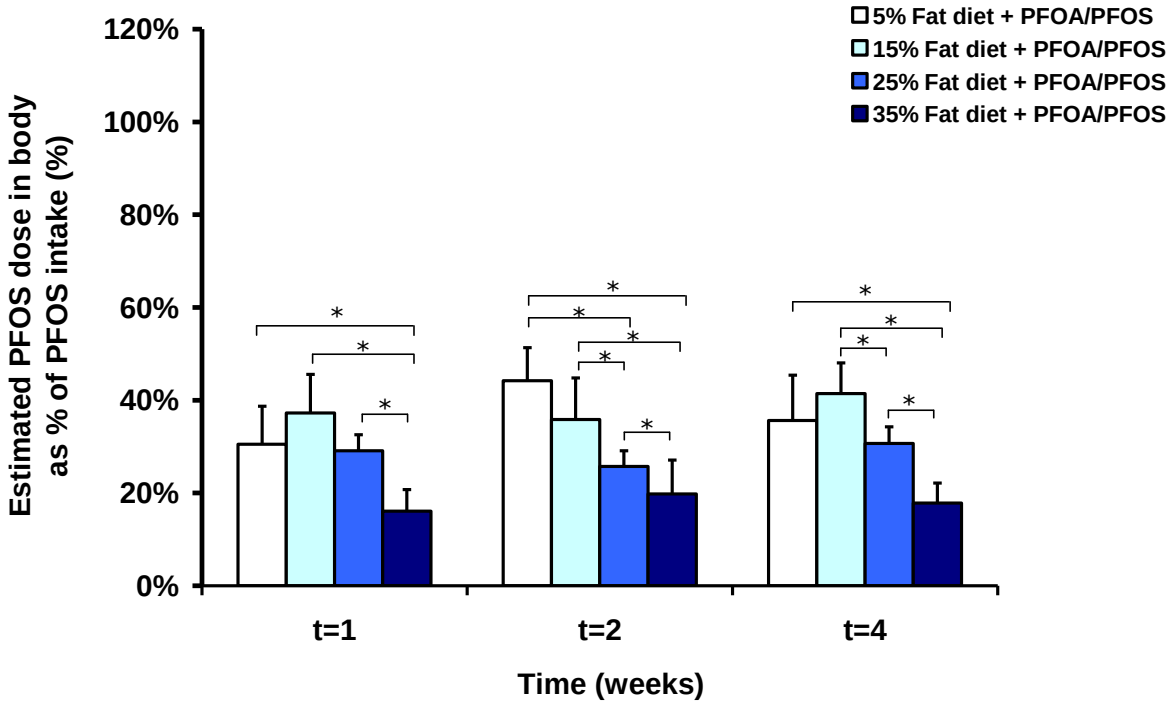
When compared to the 5% fat diet group the group on 35% fat diet significantly lowered the whole body PFOS concentration at all time points (by -47% (p=0.001), -55% (p<0.001) and -50% (p<0.001) after 1, 2 and 4 weeks of treatment; respectively).

Figure 4.7a Estimated PFOA dose in body as % of intake



*: p<0.05, Mann-Whitney U test

Figure 4.7b Estimated PFOS dose in body as % of intake



*: p<0.05, Mann-Whitney U test

4.8 Plasma total cholesterol

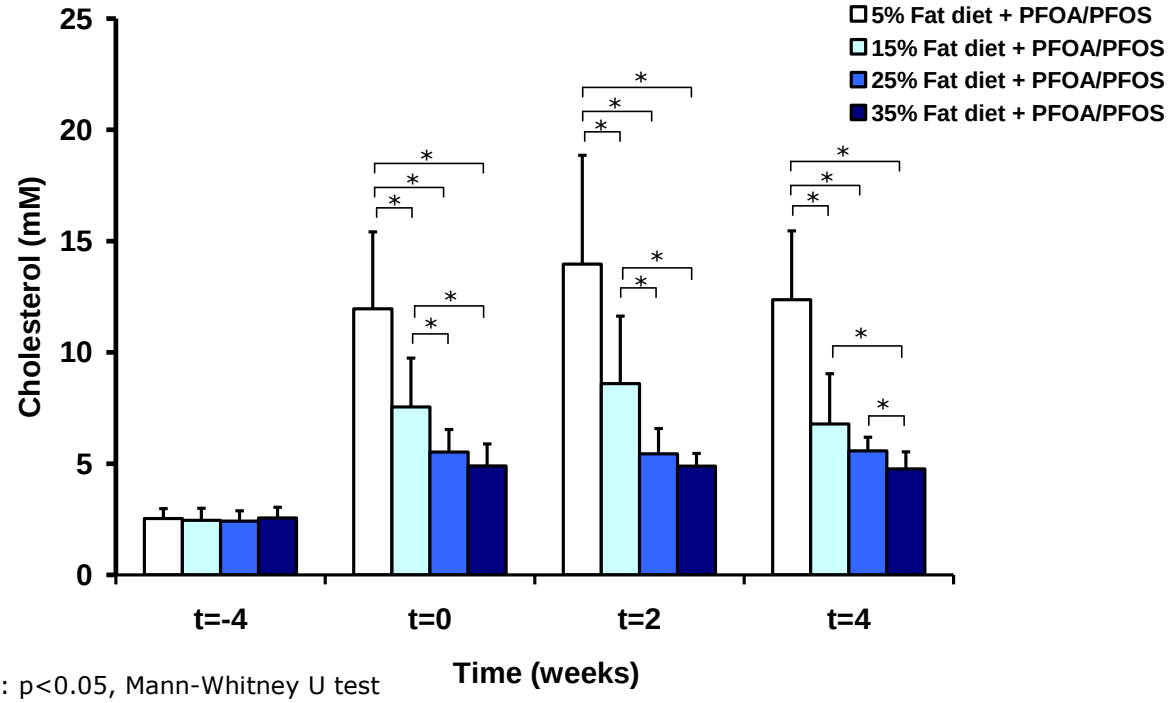
Values are absolute values (mM) and are means ± S.D. of 10 mice per group. Individual cholesterol levels per mouse and number of mice per group are given in appendix VII.

Plasma total cholesterol(mmol/L) Time (weeks)					
Group		**t=-4**	**t=0**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	2.5	12.0	14.0	12.4
	SD	0.4	3.5	4.9	3.1
15% fat + PFOA/PFOS	AVG	2.5	7.5	8.6	6.8
	SD	0.5	2.2	3.0	2.3
25% fat + PFOA/PFOS	AVG	2.4	5.5	5.4	5.6
	SD	0.5	1.0	1.1	0.6
35% fat + PFOA/PFOS	AVG	2.6	4.9	4.9	4.8
	SD	0.5	1.0	0.6	0.8

Plasma total cholesterol, P value		Time (weeks)			
		t=-4	**t=0**	**t=2**	**t=4**
Between groups		0.736	**<0.001**	**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.853	**0.005**	**0.011**	**0.001**
	25% fat + PFOA/PFOS	0.190	**<0.001**	**0.001**	**0.001**
	35% fat + PFOA/PFOS	0.912	**<0.001**	**<0.001**	**<0.001**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.971	**0.015**	**0.005**	0.393
	35% fat + PFOA/PFOS	0.739	**0.007**	**0.002**	**0.011**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.353	0.247	0.190	**0.023**

On a regular chow diet (at t=-4 weeks, before the start of the run-in period with different fat diets), plasma total cholesterol levels were similar in all groups (approximately 2.5 mM). The fat content in the diets majorly affected plasma total cholesterol levels in a fat-concentration dependent manner (ranging from approximately 12 mM for the 5% fat diet to approximately 5 mM for the 35% fat diet) . When comparing the lowest fat content diet (5% fat) with the highest (35% fat), the latter showed a robust reduction of total cholesterol levels (by -59% (p<0.001), -65% (p<0.001), and -61% (p<0.001) at t=0, 2 and 4 weeks; respectively).

Figure 4.8 Plasma total cholesterol



*: p<0.05, Mann-Whitney U test

4.9 Plasma triglycerides

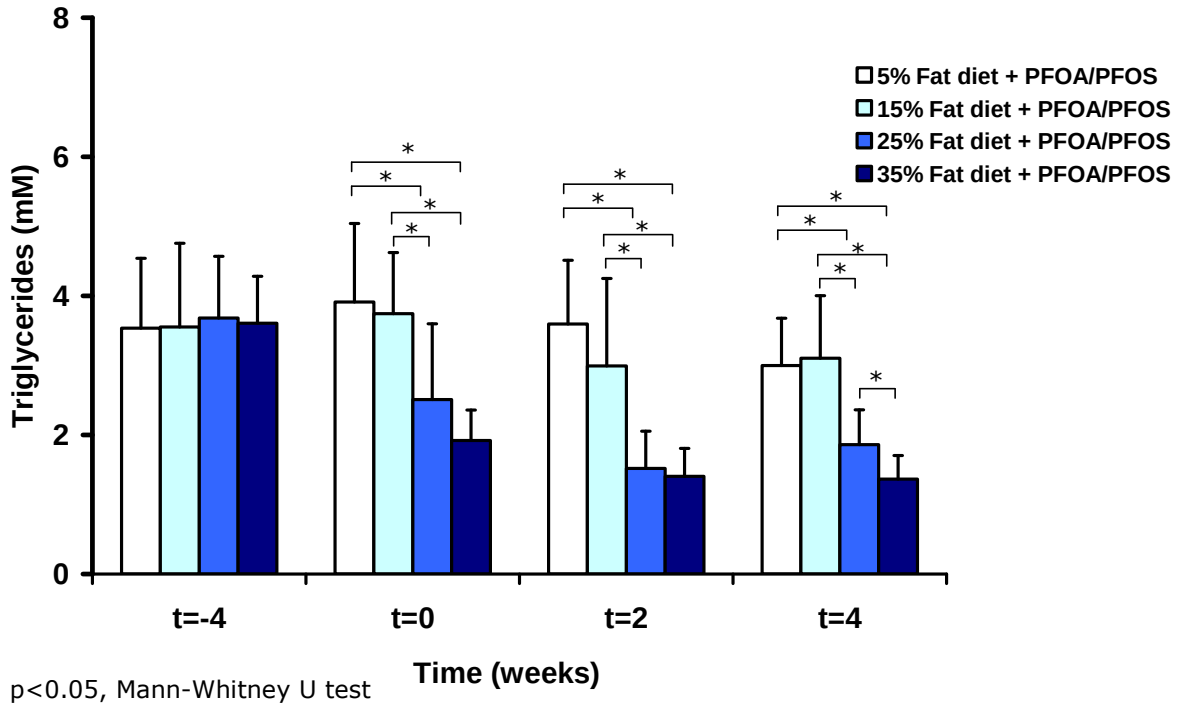
Values are absolute values (mM) and are means ± S.D. of 10 mice per group. Individual triglycerides levels per mouse and number of mice per group are given in appendix VIII.

Plasma triglycerides (mmol/L) Time (weeks)					
Group		**t=-4**	**t=0**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	3.5	3.9	3.6	3.0
	SD	1.0	1.1	0.9	0.7
15% fat + PFOA/PFOS	AVG	3.6	3.7	3.0	3.1
	SD	1.2	0.9	1.3	0.9
25% fat + PFOA/PFOS	AVG	3.7	2.5	1.5	1.9
	SD	0.9	1.1	0.5	0.5
35% fat + PFOA/PFOS	AVG	3.6	1.9	1.4	1.4
	SD	0.7	0.4	0.4	0.3

Plasma triglycerides, P value		Time (weeks)			
		t=-4	**t=0**	**t=2**	**t=4**
Between groups		0.951	**0.001**	**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.739	0.796	0.190	0.684
	25% fat + PFOA/PFOS	0.481	**0.011**	**<0.001**	**0.001**
	35% fat + PFOA/PFOS	0.481	**0.001**	**<0.001**	**<0.001**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	1.000	**0.023**	**0.007**	**0.002**
	35% fat + PFOA/PFOS	0.912	**<0.001**	**0.003**	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.971	0.143	0.436	**0.043**

On a regular chow diet (at t=-4 weeks, before the start of the run-in period with different fat diets), plasma triglycerides levels were similar in all groups. The fat content in the diets majorly affected plasma triglycerides levels in a fat-concentration dependent manner and in a similar way as plasma total cholesterol levels were affected. When comparing the lowest fat content diet (5% fat) with the highest (35% fat), the latter showed a robust reduction of triglycerides levels (by -51% (p=0.001), -61% (p<0.001), and -54% (p<0.001) at t=0, 2 and 4 weeks; respectively).

Figure 4.9 Plasma triglycerides



*: p<0.05, Mann-Whitney U test

4.10 Plasma HDL-cholesterol

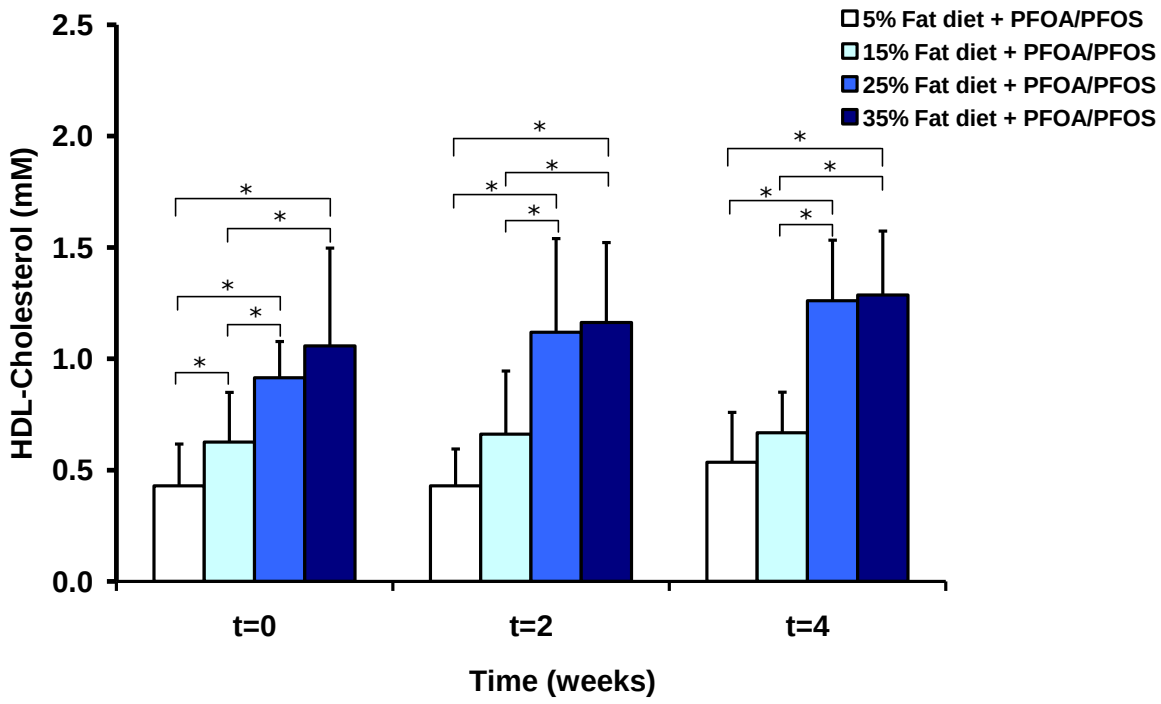
Values are absolute values (mM) and are means ± S.D. of 10 mice per group. Individual data per mouse and number of mice per group are given in appendix IX.

Plasma HDL-cholesterol(mmol/L) Time (weeks)				
Group		**t=0**	**t=2**	**t=4**
5% fat + PFOA/PFOS	AVG	0.43	0.43	0.54
	SD	0.19	0.16	0.22
15% fat + PFOA/PFOS	AVG	0.63	0.66	0.67
	SD	0.22	0.28	0.18
25% fat + PFOA/PFOS	AVG	0.91	1.12	1.26
	SD	0.16	0.42	0.27
35% fat + PFOA/PFOS	AVG	1.06	1.16	1.29
	SD	0.44	0.36	0.29

Plasma HDL-cholesterol, P value		Time (weeks)		
		t=0	**t=2**	**t=4**
Between groups		**<0.001**	**<0.001**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	**0.043**	0.075	0.165
	25% fat + PFOA/PFOS	**<0.001**	**<0.001**	**<0.001**
	35% fat + PFOA/PFOS	**0.001**	**<0.001**	**<0.001**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	**0.004**	**0.011**	**<0.001**
	35% fat + PFOA/PFOS	**0.023**	**0.005**	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.529	0.579	0.631

The fat content in the diets majorly affected plasma HDL-cholesterol levels in a fat-concentration dependent manner. When comparing the lowest fat content diet (5% fat) with the highest (35% fat), the latter showed a robust elevation of HDL-cholesterol levels (by +147% (p=0.001), +171% (p<0.001) and +140% (p<0.001) at t=0, 2 and 4 weeks; respectively).

Figure 4.10 Plasma HDL-cholesterol



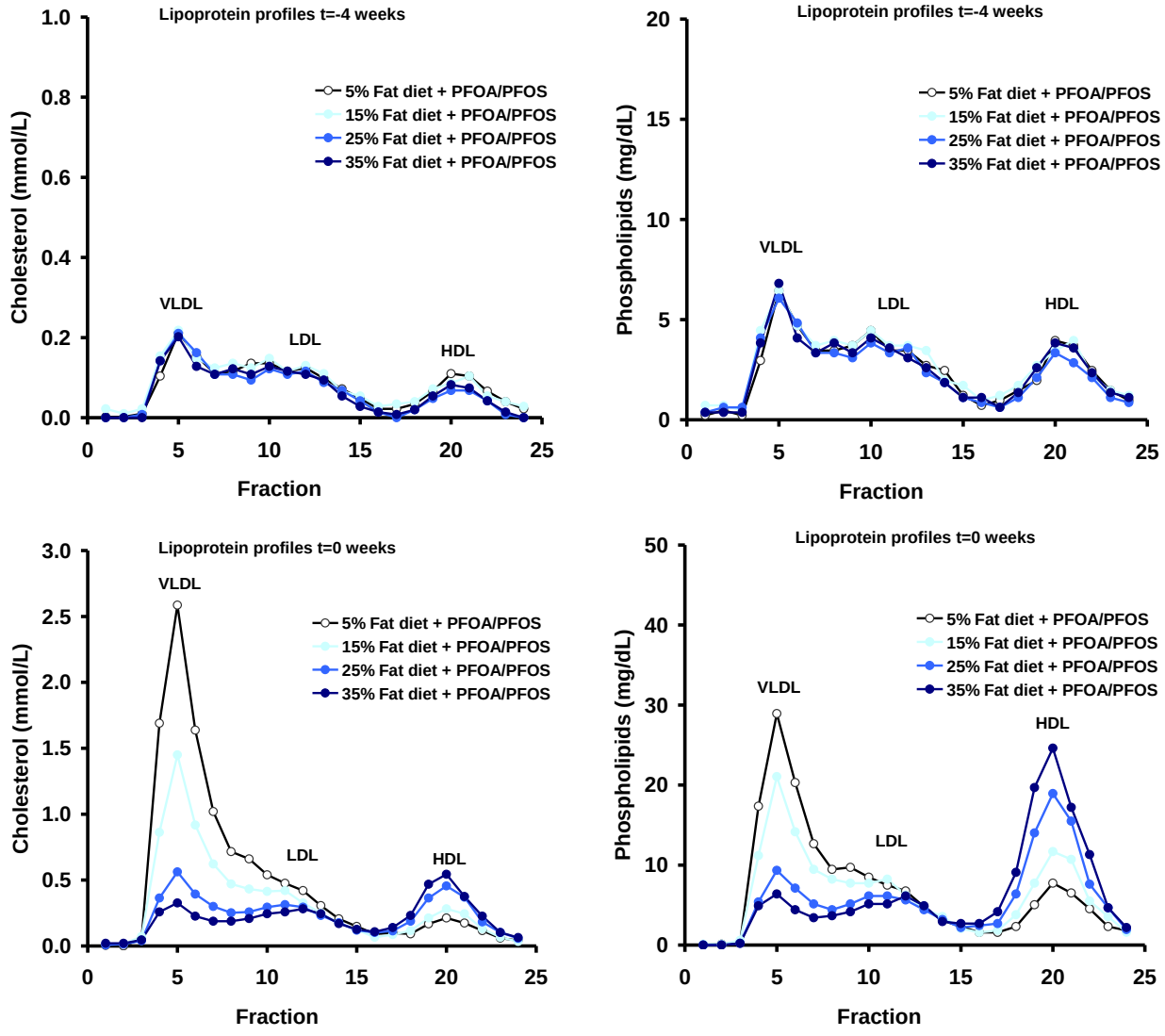
*: p<0.05, Mann-Whitney U test

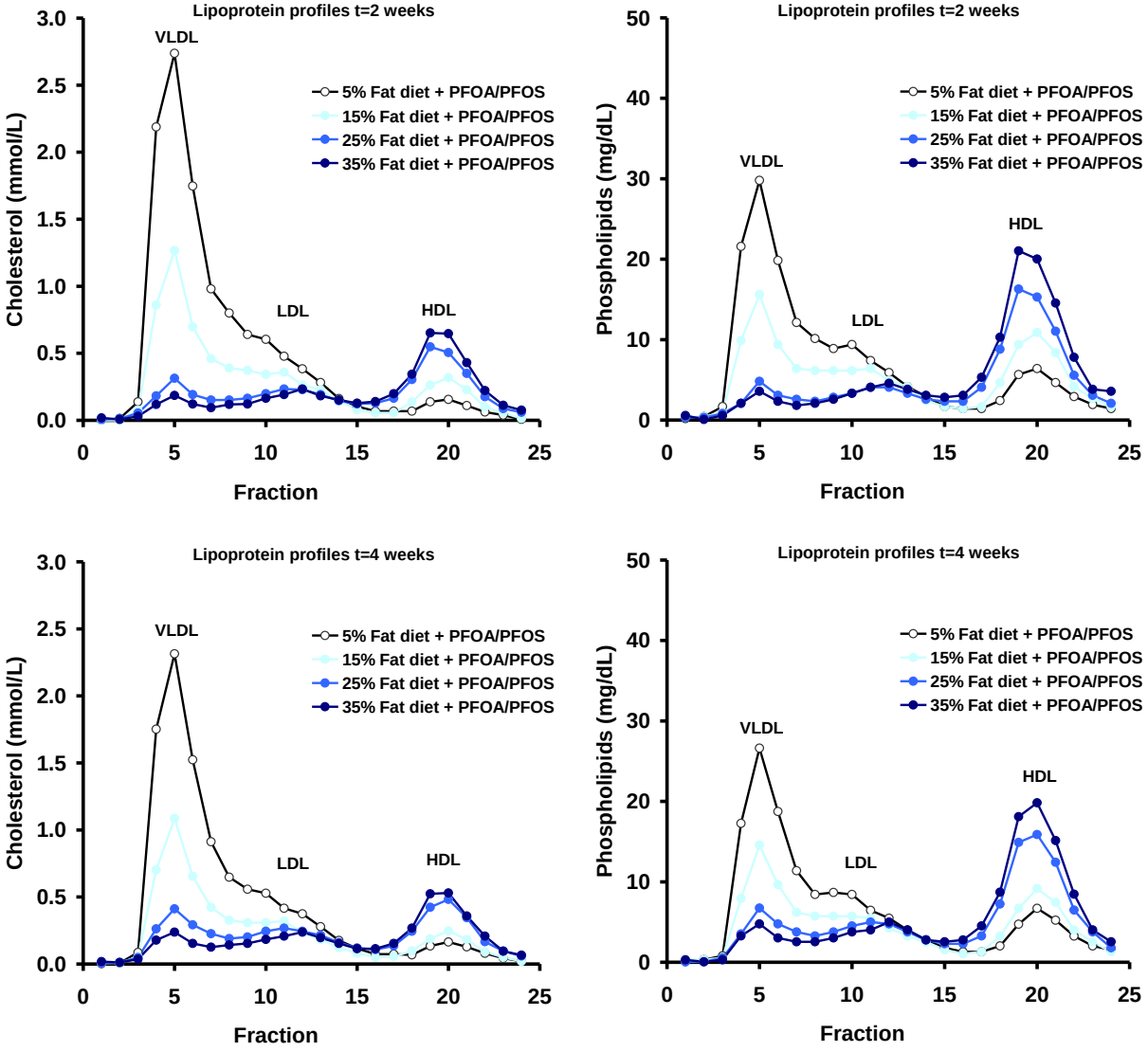
4.11 Lipoprotein profiles

Lipoproteins were separated on a Superose column. Values are absolute values (mM) from cholesterol (left graphs) and phospholipids (right graphs) measurements in pooled plasma samples per group (with 10 mice per group) at t=0 and 4 weeks. Fractions 3-8 represent VLDL, fractions 9-16 represent LDL, and fractions 17-24 represent HDL. The cholesterol and phospholipids amounts per fraction are given in appendix X.

The lipoprotein profiles were in line with the measured plasma lipid levels. At t=-4 weeks (mice were on a regular chow diet), profiles were similar between groups. The fat concentration in the diets decreased the ApoB containing lipoproteins (VLDL and LDL) and increased HDL in a fat-concentration dependent manner.

Figure 4.11 lipoprotein profiles





4.12 PFOA and PFOS liver levels

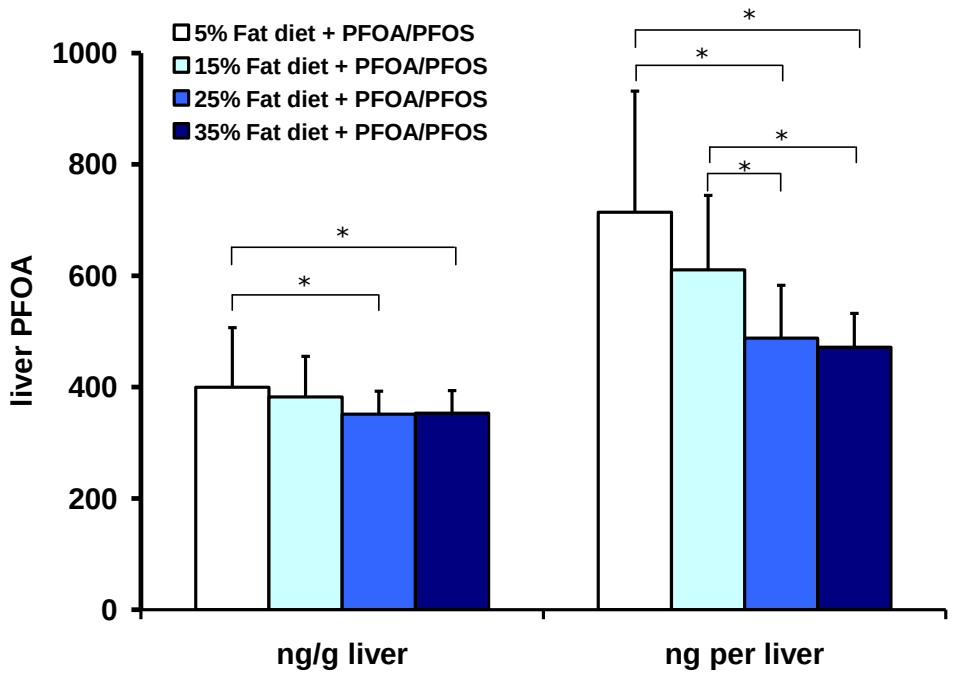
Values are absolute values (ng/g liver or ng per liver) or relative values (% of food intake) and are means ± S.D. of 10 mice per group. Individual PFOA and PFOS liver levels per mouse and number of mice per group are given in appendix XII.

PFOA and PFOS liver levels					
Group		**PFOA**		**PFOS**	
		ng/g liver	**ng per liver**	**ng/g liver**	**ng per liver**
5% fat + PFOA/PFOS	AVG	400	714	553	999
	SD	107	218	234	507
15% fat + PFOA/PFOS	AVG	382	611	478	760
	SD	73	134	202	336
25% fat + PFOA/PFOS	AVG	351	488	362	500
	SD	41	95	97	134
35% fat + PFOA/PFOS	AVG	353	471	455	608
	SD	40	61	85	124

PFOA and PFOS liver levels, P value		PFOA		PFOS	
		ng/g liver	ng per liver	ng/g liver	ng per liver
Between groups		0.061	**0.002**	0.067	**0.011**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.218	0.063	0.247	0.218
	25% fat + PFOA/PFOS	**0.043**	**0.005**	0.063	**0.011**
	35% fat + PFOA/PFOS	**0.035**	**0.004**	0.165	**0.043**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.143	**0.029**	0.105	**0.015**
	35% fat + PFOA/PFOS	0.165	**0.015**	0.912	0.165
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.684	0.579	**0.019**	0.089

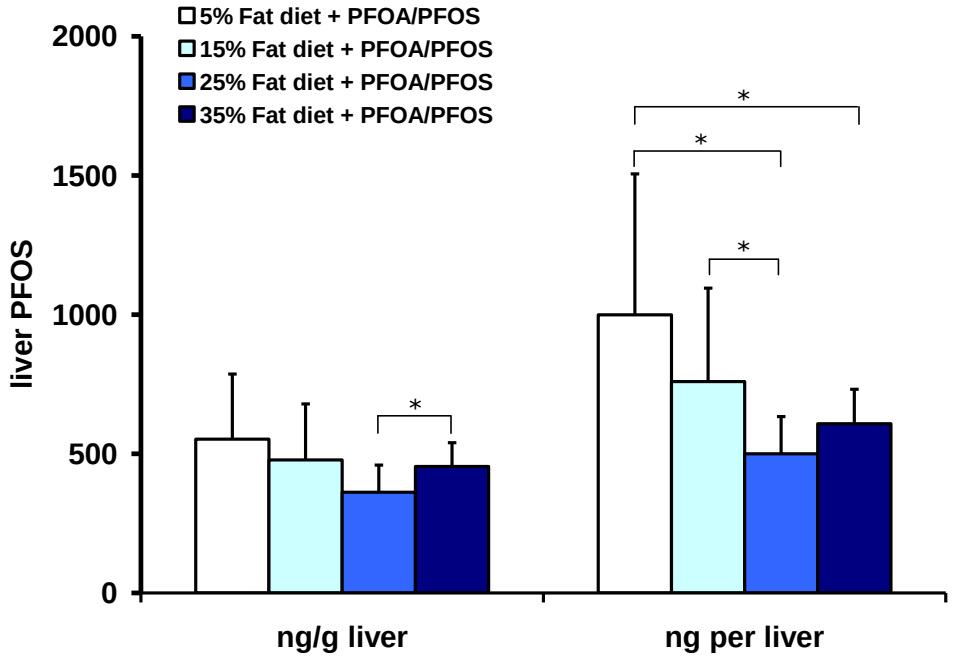
Since liver weights were significantly different between groups, PFOA and PFOS levels were also expressed as ng per liver to correct for the different liver weights. When corrected for liver weight, PFOA levels seemed to be decreased by the different fat diets in a fat-concentration dependent manner. PFOS levels, corrected for liver weight, seemed to be decreased by the different fat diets in a fat-concentration dependent manner to some extent.

Figure 4.12a Liver PFOA levels



*: p<0.05, Mann-Whitney U test

Figure 4.12b Liver PFOS levels



*: p<0.05, Mann-Whitney U test

Since the PFOA and PFOS intake was different in the different dietary fat groups, the measured liver PFOA and PFOS levels were corrected for accumulated PFOA and PFOS intake, by calculating the liver PFOA/PFOS as percentage of PFOA/PFOS intake.

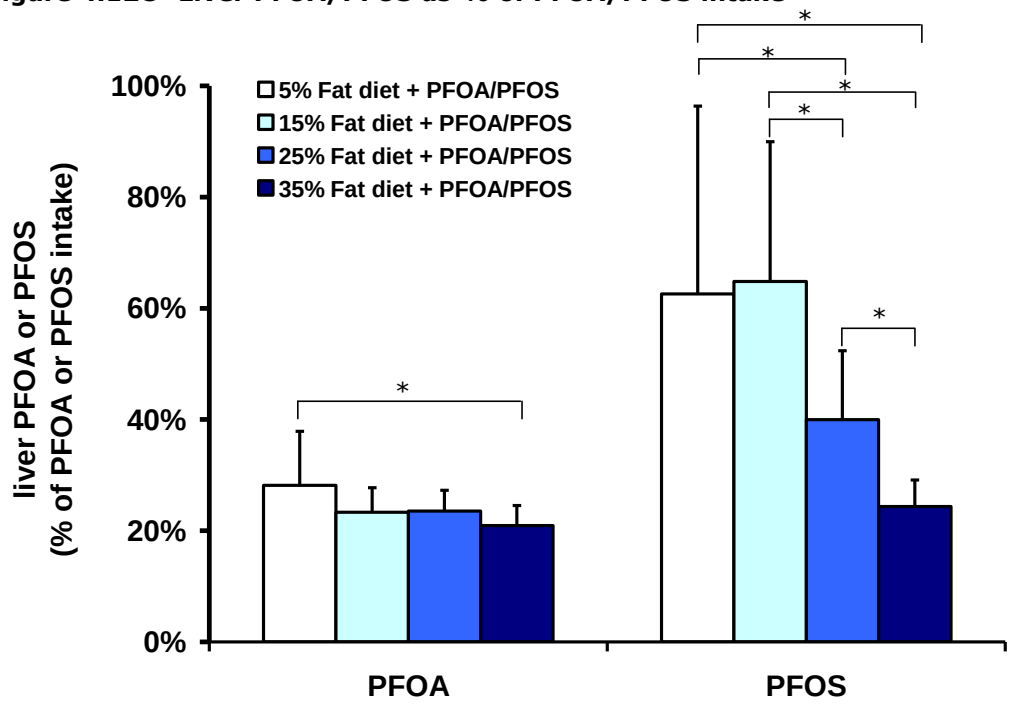
Liver PFOA and PFOS (% of PFOA and PFOS intake)			
Group		**PFOA**	**PFOS**
5% fat + PFOA/PFOS	AVG	28%	63%
	SD	10%	34%
15% fat + PFOA/PFOS	AVG	23%	65%
	SD	4%	25%
25% fat + PFOA/PFOS	AVG	24%	40%
	SD	4%	12%
35% fat + PFOA/PFOS	AVG	21%	24%
	SD	4%	5%

Liver PFOA and PFOS as % of food intake, P value		**PFOA**	**PFOS**
Between groups		**0.045**	**<0.001**
5% fat + PFOA/PFOS vs	15% fat + PFOA/PFOS	0.052	0.971
	25% fat + PFOA/PFOS	0.063	**0.043**
	35% fat + PFOA/PFOS	**0.019**	**0.003**
15% fat + PFOA/PFOS vs	25% fat + PFOA/PFOS	0.739	**0.007**
	35% fat + PFOA/PFOS	0.315	**<0.001**
25% fat + PFOA/PFOS vs	35% fat + PFOA/PFOS	0.190	**0.001**

Liver PFOA and PFOS levels as percentage of PFOA/PFOS intake, seemed to be decreased by the different fat diets in a fat-concentration dependent manner.

When compared to the 5% fat diet group the group on 35% fat diet showed a significant reduction in liver PFOA and PFOS (by -26% (p=0.019) and by -61% (p=0.003); respectively).

Figure 4.12C Liver PFOA/PFOS as % of PFOA/PFOS intake



*: p<0.05, Mann-Whitney U test

5. Conclusions

In male ApoE3L.CETP mice, the increased consumption of saturated fat led to decreased plasma total cholesterol and triglycerides levels and increased HDL-cholesterol levels. Because intake of PFOA and PFOS was not equal between mice on the different fat diets, uptake of PFOA and PFOS from the diets was estimated based on plasma PFOA and PFOS concentrations and volume of distribution compared against PFOA and PFOS intake based on diet consumption and dietary concentrations of PFOA and PFOS. The corrected estimated uptake of PFOA and PFOS levels were decreased with increasing consumption of saturated fat. Liver PFOA and PFOS levels, measured at the end of the study, confirmed the plasma results; decreased PFOA and PFOS levels with increasing consumption of saturated fat. The study indicated that in male ApoE3L.CETP mice, the increased consumption of saturated fat led to decreased PFOA and PFOS uptake and plasma concentrations while at the same time leading to decreased plasma total and non-HDL cholesterol. The results of the current study in mice do not support the hypothesis that increasing dietary fat increases the uptake and plasma concentrations of PFOA and PFOS while also increasing the plasma concentration of cholesterol.

Although the original hypothesis was not demonstrated, an important finding emerges from the experiment. Increasing dietary fat consumption in the presence of low and constant dietary concentrations of PFOA and PFOS leads not only to lower serum non-HDL cholesterol, but also lower serum PFOA and PFOS plasma concentrations, resulting from lower uptake of PFOA and PFOS. Therefore, plasma PFOA and PFOS were associated with plasma total and non-HDL cholesterol, a finding similar to that observed in epidemiological studies. The basis of these associations is not known currently, and the extent to which these experimental findings may explain the epidemiological associations observed in human populations is also not known. However, this experiment does demonstrate that a dietary factor, increasing fat, can create associations in ApoE3L.CETP mice similar to those observed in human populations in the same range of serum PFOA and PFOS concentrations.

6. Comments

In this study, male ApoE3L.CETP mice were fed four different semi-synthetic diets containing different amounts of cacao butter and sucrose. The diets contained 5%, 15%, 25% and 35% saturated fat, respectively. In previous studies, a semi-synthetic diet with 15% cacao butter and 1% corn oil (diet T) with the addition of different amounts of cholesterol has frequently been used at TNO. High-fat diets containing 24% and 35% fat have also been used previously by TNO in studies with male ApoE3L.CETP mice; however, the source of fat was lard. TNO had no prior experience in ApoE3L.CETP mice with the 5%, 25% and 35% cacao butter saturated fat diets.

As mentioned in the protocol, TNO had no prior experience with the effect of large variation of saturated fat intake on plasma lipids. The effect on plasma cholesterol of feeding mice the 5% fat diet gave especially surprising results. Plasma cholesterol levels in male ApoE3L.CETP mice fed the 5% fat diet were increased approximately 60% as compared to mice fed the 15% fat diet. TNO suspects that the amount of fast carbohydrates and saturated fat in the diet is linked to VLDL secretion. By decreasing the amount of fast carbohydrates and increasing the fat content in the diet, the VLDL secretion is reduced, and thereby, the levels of the apoB-containing lipoproteins and triglycerides are also decreased in the mice.

Treatment with the 15% fat diet (+0.25% cholesterol) resulted in plasma cholesterol levels of approximately 8 mM and triglycerides levels of approximately 3 mM, which were in line with what TNO has previously observed in male ApoE3L.CETP mice on diet T + 0.25% cholesterol (cholesterol levels of approximately 7-9 mM, triglycerides levels of approximately 2-3 mM).

The measured PFOA and PFOS concentrations in the diet deviated from the target PFOA/PFOS doses in all diets. Since the target dietary concentration of PFOA and PFOS were very low (30 ppb), we prepared the PFOA/PFOS-containing diets in 4 dilution steps. We think that these multiple dilution steps in the preparation of the PFOA/PFOS diets could have caused the deviations from the target PFOA/PFOS doses. In addition, it is possible that the distribution of PFOS and PFOA in the diets were not completely homogenous. However, all diets contained PFOA/PFOS levels in the ppb range, which was not expected to affect plasma lipid levels based on prior toxicological studies. Because the dietary concentrations of PFOA and PFOS in the different diets were somewhat variable, and in order to correct for potential differences in diet consumption, we used the dietary

concentrations of PFOA and PFOS along with the individual diet consumption as a measure of dietary intake of PFOA and PFOS and compared the dietary intake of PFOA and PFOS to the estimated uptake to the body, the latter being estimated by multiplying the plasma PFOA and PFOS concentrations with volume of distribution. This allowed us to estimate the proportions of the dietary intake of PFOA and PFOS that were absorbed.

It should be noted that:
- Food intake could not be precisely measured per mouse at all time points and with all diets.
- There was substantial variation in the levels of PFOA and PFOS measured in the diet, possibly due to non-homogeneity.
- There are some potential outliers in the measurement of plasma and liver PFOA and PFOS levels. Based on Chauvenet's criterion, 3 of 120 PFOA plasma determinations, 9 of 120 PFOS plasma determinations, 2 of 40 PFOA liver determinations and 3 of 40 PFOS liver determinations may have been outliers, which may be indicative of "hotspots" in the diet.

The current study indicated that in male ApoE3L.CETP mice, after correcting for PFOA and PFOS intake, estimated uptakes of PFOA and PFOS from the diet were decreased with increasing consumption of saturated fat. Although the question remains whether the results in ApoE3L.CETP mice can be extrapolated to the human situation, the current study in mice does not support the hypothesis that increased consumption of saturated fats lead to increased serum/plasma concentrations of PFOA and PFOS as well as increased serum/plasma cholesterol concentration.

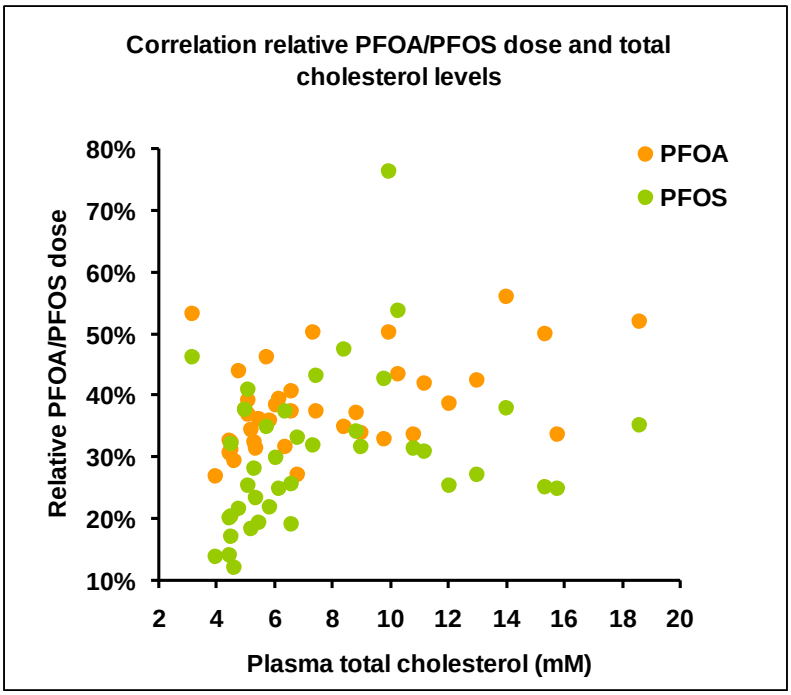
The finding in the current study that the uptakes of PFOA and PFOS were decreased with increasing consumption of saturated fat may be related to a decreased intestinal absorption of dietary PFOA and PFOS with increasing consumption of saturated fat. Perhaps the increased fat content in the diet caused a less efficient intestinal fat absorption that coincided with decreased absorption of PFOA and PFOS. The observations that the mice consumed more of the higher fat diets than what we would expect based on an isocaloric intake and that the mice fed the higher fat diets did not gain more weight than the low fat group are consistent with less efficient intestinal fat absorption when fed the higher fat diets. It is possible that at higher fat intakes the potentially reduced fat uptake and consequently increased fecal excretion carried away PFOA and PFOS. Unfortunately, we did not collect fecal samples in the current study to examine the fecal excretion of fatty acids and PFOA/PFOS.

Increasing consumption of saturated fat could also affect PFOA and PFOS storage in various tissues, leading to decreased plasma PFOA and PFOS levels. In the current study, liver weights were decreased significantly by the increasing fat diets. PFOA and PFOS are not known to distribute into fat, and fat tissues were not evaluated at the end of the study. In order to evaluate the effect of saturated fat consumption on PFOA and PFOS storage in liver, PFOA and PFOS liver levels were measured. The liver levels confirmed the plasma results; decreased PFOA and PFOS levels with increasing consumption of saturated fat.

Another possible explanation for the observed decreased PFOA and PFOS levels with increasing consumption of saturated fat could lie in the results of the different fat diets on plasma lipid levels. In male ApoE3L.CETP mice, the increased consumption of saturated fat and decreased consumption of fast carbohydrates led to decreased plasma total cholesterol levels. The estimated PFOA and PFOS intake levels and plasma concentrations were also decreased with increased dietary fat and decreased fast carbohydrates. Because positive associations of serum PFOA or PFOS with serum non-HLD cholesterol have been observed in epidemiological studies, we have tested whether there is a significant correlation between PFOA/PFOS levels and plasma total cholesterol levels.

Per mouse the average estimated PFOA and PFOS levels during treatment (t=1-4 weeks) and the average cholesterol levels (t=0-4 weeks) were calculated (see appendix XI for individual values).

Significance of correlation between parameters were calculated non-parametrically, using the computer program SPSS. The Spearman's rho non-parametric correlations algorithm was used for the calculation of the correlation coefficient and the significance.



Correlation	Spearman's rho correlation coefficient	P value
PFOA dose-cholesterol	0.445	0.004
PFOS dose-cholesterol	0.417	0.008

In the current study, there is a significant positive correlation between both the PFOA levels and plasma cholesterol and the PFOS levels and plasma cholesterol. The increase in PFOA and PFOS levels could be caused by the increase in plasma cholesterol by a yet unknown mechanism.

To exclude that the increase in PFOA/PFOS levels and the increase in plasma cholesterol levels are two independent effects, both caused by the dietary fat content, ApoE3L.CETP mice could be fed diets with the same dietary fat and PFOA/PFOS content but with different dietary cholesterol concentrations. To investigate what the effect of a diet containing fibers and plant sterols next to a low fat is on PFOA and PFOS plasma levels, a group fed a regular chow diet could also be added in the same experiment.

Taking into account that the current study has been performed in mice, the results do not support the hypothesis that the observed association between serum PFOA or PFOS levels and serum total cholesterol in humans is the result of an increased consumption of saturated fat. Perhaps by only changing the fat and sucrose content in the diets, we have not taken into account that in humans the increased consumption of saturated fat could coincide with an increased consumption of dietary cholesterol and could also coincide with a decreased consumption of dietary fibers and plant sterols (for example by eating a meat-rich and vegetable-poor diet). In studies performed at TNO, it has been observed that the intestinal absorption of lipophilic compounds was higher in a diet with a higher cholesterol concentration and higher in a diet with lower dietary fiber and plant sterol content.

It is quite possible that feeding mice higher dietary cholesterol leads to a high bile acid production and a higher biliary pool, causing a higher intestinal absorption of the amphiphilic PFOA and PFOS. The addition of fibers and plant sterols leads to a lower intestinal cholesterol absorption and perhaps also to a lower intestinal absorption of PFOA and PFOS.

In the proposed study, the effect of dietary cholesterol on plasma PFOA and PFOS levels (and PFOA and PFOS fecal excretion) and the effect of a chow diet (high on dietary fiber and plant sterol

content) versus a semi synthetic fat diet on plasma PFOA and PFOS levels (and PFOA and PFOS fecal excretion) could be examined.

A further limitation of the present study was that a fat composition was used which does not represent the composition of dietary fat in humans. The present fat is very rich in saturated fat with lower amounts of monounsaturated fat and a low amount of polyunsaturated fat. In man much more mono- and polyunsaturated fat is consumed. Moreover, the fat intake lies in general between 25 and 45 kcal%. In the present study, the range was 12 – 64 kcal%.

Therefore, a suggestion for the proposed study is to use a westernized diet instead of a diet in which the main fat source is cacoa butter. In westernized diet the dietary fat composition has been humanized (with 45% saturated fat, 35% mono unsaturated fat and 20% poly unsaturated fat and an energy of 37kcal% fat), but the fat sources are not from animal source (the humanized fat contains soy bean oil, olive oil, cacoa butter, coconut oil and palm oil).

7. References

1. Van den Maagdenberg AMJM, Hofker MH, Krimpenfort PJA, de Bruijn IG, van Vlijmen B, van der Boom H, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. **J Biol Chem 1993**; 268: 10540-10545.
2. Van Vlijmen B, van den Maagdenberg AMJM, Gijbels MJJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. **J Clin Invest 1994**; 93: 1403-1410.
3. Groot PHE, van Vlijmen BJM, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, Havekes LM. Quantitative assessment of aortic atherosclerosis in apoE3Leiden transgenic mice and its relationship to serum cholesterol exposure. **Arterioscler Thromb Vasc Biol 1996**; 16: 926-933.
4. Zadelaar S, Kleemann R, Verschuren L, de Vries-van der Weij J, van der Hoorn J, Princen HM, Kooistra T. Mouse models for athereosclerosis and pharmaceutical modifiers. **Arterioscler Thromb Vasc Biol 2007:** 27; 1706-1721.
5. Westerterp M, Van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM, Rensen PCN. Cholesteryl ester transfer protein decreases HDL and severely aggravates atherosclerosis in APOE*3Leiden mice. **Arterioscler Thromb Vasc Biol 2006:** 26; 2552-2559.
6. Van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HMG, Jukema JW, Havekes LM, Rensen PCN. Fenofibrate increases HDL cholesterol by reducing cholesteryl ester transfer protein expression. **J Lipid Res 2007;** 48: 1763-1771.
7. De Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HMG, Romijn JA, Jukema JW, Havekes LM, Rensen PCN. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. **Atherosclerosis 2008;** 197: 57-63.
8. Van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC, Princen HM Niacin Increases HDL by Reducing Hepatic Expression and Plasma Levels of Cholesteryl Ester Transfer Protein in APOE*3Leiden.CETP Mice. **Arterioscler Thromb Vasc Biol 2008;** 28: 2016-2022.
9. De Haan W., de Vries-Van der Weij J, van der Hoorn JWA, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HMG, Rensen PCN. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. **Circulation 2008;** 117: 2515-2522.

8. Appendices

Appendix I Body weight

Group 1: 5% fat diet + PFOA/PFOS

Mouse#	Cage#	Body weight (g) in time			
		t=-4	t=0	t=2	t=4
1	1	24.4	27.1	27.8	28.5
2	1	27.1	28.7	28.6	28.7
3	1	25.7	28.7	29.4	30.1
4	2	25.3	27.0	27.3	28.5
5	2	20.9	23.8	25.1	25.7
6	3	24.6	26.4	27.8	27.2
7	3	25.4	26.6	27.4	27.8
8	4	26.3	29.3	29.8	30.3
9	4	26.6	29.7	30.8	31.1
10	4	30.4	33.4	33.8	33.0
AVG		**25.7**	**28.1**	**28.8**	**29.1**
SD		**2.4**	**2.6**	**2.4**	**2.1**
n		**10**	**10**	**10**	**10**

Group 2: 15% fat diet + PFOA/PFOS

Mouse#	Cage#	Body weight (g) in time			
		t=-4	t=0	t=2	t=4
11	5	23.1	25.7	25.6	26.8
12	5	25.2	27.2	27.9	28.2
13	5	27.3	29.8	30.9	31.9
14	6	20.2	22.5	22.4	22.9
15	6	24.4	27.4	28.8	30.2
16	7	27.8	31.5	33.2	34.2
17	7	26.0	29.9	30.8	33.1
18	8	26.4	29.8	29.6	30.2
19	8	27.8	30.3	31.4	30.9
20	8	27.5	29.0	30.0	30.3
AVG		**25.6**	**28.3**	**29.1**	**29.9**
SD		**2.4**	**2.7**	**3.1**	**3.3**
n		**10**	**10**	**10**	**10**

Group 3: 25% fat diet + PFOA/PFOS

Mouse#	Cage#	Body weight (g) in time			
		t=-4	t=0	t=2	t=4
21	9	25.2	26.4	26.4	27.4
22	9	24.4	27.3	28.9	30.4
23	9	25.3	27.1	27.8	28.0
24	9	25.2	27.2	27.4	28.0
25	10	25.6	26.9	26.4	26.6
26	10	23.3	24.4	24.1	24.3
27	11	28.7	29.2	28.8	30.2
28	11	26.7	28.0	28.7	29.2
29	12	23.3	26.5	26.8	28.1
30	12	23.1	24.5	25.7	26.3
AVG		**25.1**	**26.8**	**27.1**	**27.9**
SD		**1.7**	**1.5**	**1.5**	**1.8**
n		**10**	**10**	**10**	**10**

Group 4: 35% fat diet + PFOA/PFOS

Mouse#	Cage#	Body weight (g) in time			
		t=-4	t=0	t=2	t=4
31	13	24.2	26.4	26.8	27.4
32	13	24.3	27.0	27.7	28.2
33	14	22.6	24.1	25.5	25.3
34	14	25.0	26.9	28.9	29.5
35	15	28.3	29.6	30.1	30.6
36	15	26.6	29.1	30.2	31.1
37	16	25.1	29.3	31.0	32.6
38	16	23.2	26.0	26.2	26.5
39	17	25.5	26.8	27.3	27.3
40	17	26.0	26.8	27.0	27.8
AVG		**25.1**	**27.2**	**28.1**	**28.6**
SD		**1.7**	**1.7**	**1.9**	**2.3**
n		**10**	**10**	**10**	**10**

Appendix II Liver weight

Group 1: 5% fat diet + PFOA/PFOS

Mouse#	Cage#	Liver weight (g) t=4 weeks
1	1	1.71
2	1	2.08
3	1	2.12
4	2	1.80
5	2	1.76
6	3	1.65
7	3	1.70
8	4	1.72
9	4	1.23
10	4	1.87
AVG		**1.8**
SD		**0.2**
n		**10**

Group 2: 15% fat diet + PFOA/PFOS

Mouse#	Cage#	Liver weight (g) t=4 weeks
11	5	1.35
12	5	1.68
13	5	1.90
14	6	1.17
15	6	1.71
16	7	1.65
17	7	1.87
18	8	1.58
19	8	1.68
20	8	1.47
AVG		**1.6**
SD		**0.2**
n		**10**

Group 3: 25% fat diet + PFOA/PFOS

Mouse#	Cage#	Liver weight (g) t=4 weeks
21	9	1.34
22	9	1.52
23	9	1.44
24	9	1.38
25	10	1.37
26	10	1.29
27	11	1.52
28	11	1.50
29	12	1.27
30	12	1.16
AVG		**1.4**
SD		**0.1**
n		**10**

Group 4: 35% fat diet + PFOA/PFOS

Mouse#	Cage#	Liver weight (g) t=4 weeks
31	13	1.35
32	13	1.32
33	14	1.17
34	14	1.28
35	15	1.45
36	15	1.53
37	16	1.36
38	16	1.26
39	17	1.29
40	17	1.35
AVG		**1.3**
SD		**0.1**
n		**10**

Appendix III Food and fat intake

Group 1: 5% fat diet + PFOA/PFOS

Cage#	Number of mice	Food intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
1	3	3.42	3.48	3.39	3.26	3.21	3.30	3.10
2	2	3.51	3.73	3.58	3.65	3.48	3.75	3.56
3	2	3.16	3.35	3.02	2.94	2.94	2.74	2.91
4	3	4.28	3.95	4.06	4.23	4.42	4.40	4.06
AVG		**3.6**	**3.6**	**3.5**	**3.5**	**3.5**	**3.5**	**3.4**
SD		**0.5**	**0.3**	**0.4**	**0.6**	**0.6**	**0.7**	**0.5**
n		**4**	**4**	**4**	**4**	**4**	**4**	**4**

Group 2: 15% fat diet + PFOA/PFOS

Cage#	Number of mice	Food intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
5	3	3.54	3.32	3.71**	3.86**	3.83**	3.94**	3.96**
6	2	3.28	3.38	3.25	3.20	3.21	3.19	2.91
7	2	*	3.89	3.37**	3.38**	3.85**	3.06**	3.62**
8	3	*	*	3.82**	3.94**	3.90**	3.86**	4.01**
AVG		**3.4**	**3.5**	**3.5**	**3.6**	**3.7**	**3.5**	**3.6**
SD/Range		**0.1**	**0.3**	**0.3**	**0.4**	**0.3**	**0.5**	**0.5**
n		**2**	**3**	**4**	**4**	**4**	**4**	**4**

Group 3: 25% fat diet + PFOA/PFOS

Mouse#	Cage#	Food intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
9	4	3.33	2.95	3.09**	3.25**	3.24**	3.14**	3.38**
10	2	2.91	2.96	2.87	2.71	2.78	2.76	2.73
11B	1	*	3.93	3.63	3.50	3.12	3.39	3.47
11A	1		3.44	3.03	3.12	3.18	3.47	3.19
12	2	*	*	3.08	3.08	3.21	3.11	3.16**
AVG		**3.1**	**3.3**	**3.1**	**3.1**	**3.1**	**3.2**	**3.2**
SD/Range		**0.2**	**0.5**	**0.3**	**0.3**	**0.2**	**0.3**	**0.3**
n		**2**	**4**	**5**	**5**	**5**	**5**	**5**

Mouse from cage 11 were split in week -3, because of fighting wounds

Group 4: 35% fat diet + PFOA/PFOS

Mouse#	Cage#	Food intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
13	2	3.04	3.30	3.13	3.15	3.09	3.10	2.89
14	2	3.00	2.50	2.73	3.03	3.01	2.73	3.12**
15	2	3.13	2.93	3.23	3.19	3.33	3.37	3.16
16	2	*	3.21	3.42**	3.73**	3.52**	4.12**	4.01**
17	2	3.18	*	3.13**	3.43**	3.84**	3.57**	3.52**
AVG		**3.1**	**3.0**	**3.1**	**3.3**	**3.4**	**3.4**	**3.3**
SD		**0.1**	**0.4**	**0.3**	**0.3**	**0.3**	**0.5**	**0.4**
n		**4**	**4**	**5**	**5**	**5**	**5**	**5**

*: a lot of small crums on bottom of cage, food intake was not measured accurately
**: mice crumbled food, crums on bottom of cage were also collected for accurate food intake measurements

Group 1: 5% fat diet + PFOA/PFOS

Cage#	Number of mice	Fat intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
1	3	0.17	0.17	0.17	0.16	0.16	0.16	0.15
2	2	0.18	0.19	0.18	0.18	0.17	0.19	0.18
3	2	0.16	0.17	0.15	0.15	0.15	0.14	0.15
4	3	0.21	0.20	0.20	0.21	0.22	0.22	0.20
AVG		**0.18**	**0.18**	**0.18**	**0.18**	**0.18**	**0.18**	**0.17**
SD		**0.02**	**0.01**	**0.02**	**0.03**	**0.03**	**0.04**	**0.03**
n		**4**	**4**	**4**	**4**	**4**	**4**	**4**

Group 2: 15% fat diet + PFOA/PFOS

Cage#	Number of mice	Fat intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
5	3	0.53	0.50	0.56**	0.58**	0.58**	0.59**	0.59**
6	2	0.49	0.51	0.49	0.48	0.48	0.48	0.44
7	2	*	0.58	0.51**	0.51**	0.58**	0.46**	0.54**
8	3	*	*	0.57**	0.59**	0.59**	0.58**	0.60**
AVG		**0.51**	**0.53**	**0.53**	**0.54**	**0.55**	**0.53**	**0.54**
SD/Range		**0.02**	**0.05**	**0.04**	**0.05**	**0.05**	**0.07**	**0.08**
n		**2**	**3**	**4**	**4**	**4**	**4**	**4**

Group 3: 25% fat diet + PFOA/PFOS

Mouse#	Cage#	Fat intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
9	4	0.83	0.74	0.77**	0.81**	0.81**	0.79**	0.85**
10	2	0.73	0.74	0.72	0.68	0.69	0.69	0.68
11B	1	*	0.98	0.91	0.88	0.78	0.85	0.87
11A	1		0.86	0.76	0.78	0.80	0.87	0.80
12	2	*	*	0.77	0.77	0.80	0.78	0.79**
AVG		**0.78**	**0.83**	**0.79**	**0.78**	**0.78**	**0.79**	**0.80**
SD/Range		**0.05**	**0.12**	**0.07**	**0.07**	**0.05**	**0.07**	**0.07**
n		**2**	**4**	**5**	**5**	**5**	**5**	**5**

Mouse from cage 11 were split in week -3, because of fighting wounds

Group 4: 35% fat diet + PFOA/PFOS

Mouse#	Cage#	Fat intake (g/day/mouse) in time						
		t=-3	t=-2	t=0	t=1	t=2	t=3	t=4
13	2	1.06	1.16	1.10	1.10	1.08	1.09	1.01
14	2	1.05	0.88	0.96	1.06	1.05	0.96	1.09**
15	2	1.09	1.02	1.13	1.12	1.17	1.18	1.11
16	2	*	1.12	1.20**	1.30**	1.23**	1.44**	1.41**
17	2	1.11	*	1.10**	1.20**	1.34**	1.25**	1.23**
AVG		**1.08**	**1.04**	**1.10**	**1.16**	**1.18**	**1.18**	**1.17**
SD		**0.03**	**0.13**	**0.09**	**0.10**	**0.12**	**0.18**	**0.15**
n		**4**	**4**	**5**	**5**	**5**	**5**	**5**

*: a lot of small crums on bottom of cage, food intake was not measured accurately

**: mice crumbled food, crums on bottom of cage were also collected for accurate food intake measurements

Appendix IV Accumulated PFOA and PFOS intake

Aimed and measured PFOA/PFOS doses in diets

Measured PFOA/PFOS doses in regular chow diet and different fat diets without PFOA/PFOS added, were below detection limit (<0.5 ppb).

PFOA/PFOS in diet preparation

Weighed:	0.1249 g PFOS 0.1250 g PFOA	weighed PFOS mixed with weighed PFOA	
3 parts per thousand PFOA/PFOS:		0.125 g PFOA/PFOS in	41.7 g 5% fat diet
0.3 parts per thousand PFOA/PFOS:		10 gram 3 parts per thousand PFOA/PFOS +	90 g 5% fat diet
3 ppm PFOA/PFOS:		10 gram 0.3 parts per thousand PFOA/PFOS +	990 g 5% fat diet

35% fat diet: 30 ppb PFOA/PFOS 60 g 3 ppm PFOA/PFOS mixed in 6 kg 35% fat diet
PFOA/FPOS amount in other diets based on food intake t=-3/t=-2 (equal dosing as 35% fat diet)

Food intake t=-3/t=-2:

Group	Food intake (g/d/mouse)
Group 1: 5% fat diet	3.61
Group 2: 15% fat diet	3.47
Group 3: 25% fat diet	3.22
Group 4: 35% fat diet	3.03

			Aimed PFOA/PFOS
		mixed in 6 kg	in diet (ppb)
60.0	gram 3 ppm PFOA+ PFOS	35% fat diet	30
56.5	gram 3 ppm PFOA+ PFOS	25% fat diet	32
52.5	gram 3 ppm PFOA+ PFOS	15% fat diet	34
50.4	gram 3 ppm PFOA+ PFOS	5% fat diet	36

Measured PFOA/PFOS in diet

Group	Measured PFOA in diet ppb	Average PFOA in diet ppb	Measured PFOS in diet ppb	Average PFOS in diet ppb
Group 1: 5% fat diet	24.3 32.5	28.4	20.6 15.0	17.8
Group 2: 15% fat diet	26.1 28.6	27.4	10.8 13.4	12.1
Group 3: 25% fat diet	27.0 23.7	25.3	17.5 13.4	15.5
Group 4: 35% fat diet	20.7 31.7	26.2	37.4 20.3	28.8

Group 1: 5% fat diet

Mouse#	Cage#	Accumulated PFOA intake (ng) in time			
		t=1	t=2	t=3	t=4
1	1	555	1102	1757	2373
2	1	555	1102	1757	2373
3	1	555	1102	1757	2373
4	2	622	1215	1961	2669
5	2	622	1215	1961	2669
6	3	502	1003	1547	2127
7	3	502	1003	1547	2127
8	4	722	1475	2349	3156
9	4	722	1475	2349	3156
10	4	722	1475	2349	3156
AVG		**608**	**1217**	**1934**	**2618**
SD		**88**	**192**	**318**	**413**
n		**10**	**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	Accumulated PFOA intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
1	1	20.2	39.6	83.3
2	1	19.4	38.5	82.7
3	1	19.1	37.5	78.8
4	2	22.9	44.5	93.6
5	2	25.5	48.4	103.8
6	3	18.5	36.1	78.2
7	3	18.6	36.6	76.5
8	4	24.4	49.5	104.2
9	4	23.9	47.9	101.5
10	4	21.5	43.6	95.6
AVG		**21**	**42**	**90**
SD		**3**	**5**	**11**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Accumulated PFOA intake (ng) in time			
		t=1	t=2	t=3	t=4
11	5	633	1263	2017	2776
12	5	633	1263	2017	2776
13	5	633	1263	2017	2776
14	6	525	1052	1663	2220
15	6	525	1052	1663	2220
16	7	556	1188	1774	2467
17	7	556	1188	1774	2467
18	8	648	1288	2028	2797
19	8	648	1288	2028	2797
20	8	648	1288	2028	2797
AVG		**600**	**1213**	**1901**	**2609**
SD		**53**	**93**	**162**	**243**
n		**10**	**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Accumulated PFOA intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
11	5	24.7	49.3	103.6
12	5	23.0	45.3	98.4
13	5	20.9	40.9	87.0
14	6	23.4	47.0	96.9
15	6	18.7	36.5	73.5
16	7	17.2	35.8	72.1
17	7	18.3	38.6	74.5
18	8	21.8	43.5	92.6
19	8	21.0	41.0	90.5
20	8	22.0	42.9	92.3
AVG		**21**	**42**	**88**
SD		**2**	**4**	**11**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Accumulated PFOA intake (ng) in time			
		t=1	t=2	t=3	t=4
21	9	495	987	1544	2144
22	9	495	987	1544	2144
23	9	495	987	1544	2144
24	9	495	987	1544	2144
25	10	412	833	1323	1807
26	10	412	833	1323	1807
27	11B	532	1006	1606	2221
28	11A	474	957	1573	2138
29	12	469	956	1508	2068
30	12	469	956	1508	2068
AVG		**474**	**949**	**1502**	**2069**
SD		**38**	**63**	**98**	**144**
n		**10**	**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Accumulated PFOA intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
21	9	18.7	37.4	78.3
22	9	17.6	34.2	70.5
23	9	18.0	35.5	76.6
24	9	18.1	36.0	76.6
25	10	15.4	31.6	67.9
26	10	17.0	34.6	74.4
27	11B	18.3	34.9	73.6
28	11A	16.7	33.4	73.2
29	12	17.6	35.7	73.6
30	12	18.7	37.2	78.6
AVG		**18**	**35**	**74**
SD		**1**	**2**	**3**
n		**10**	**10**	**10**

3M-06: Effect of increasing amounts of dietary fat on plasma concentrations of cholesterol, PFOS, and PFOA plasma in male APOE*3-Leiden.CETP mice fed approximately 0.03 ppm of PFOS and PFOA – 07-Apr-2016

Group 4: 35% fat diet

Mouse#	Cage#	Accumulated PFOA intake (ng) in time			
		t=1	t=2	t=3	t=4
31	13	495	980	1548	2077
32	13	495	980	1548	2077
33	14	476	949	1449	2021
34	14	476	949	1449	2021
35	15	501	1025	1643	2222
36	15	501	1025	1643	2222
37	16	585	1137	1893	2628
38	16	585	1137	1893	2628
39	17	538	1141	1796	2441
40	17	538	1141	1796	2441
AVG		**519**	**1047**	**1666**	**2278**
SD		**41**	**84**	**170**	**240**
n		**10**	**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Accumulated PFOA intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
31	13	18.6	36.6	75.8
32	13	18.1	35.4	73.7
33	14	19.2	37.2	79.9
34	14	17.1	32.8	68.5
35	15	16.8	34.0	72.6
36	15	16.9	33.9	71.5
37	16	19.4	36.7	80.6
38	16	22.4	43.4	99.2
39	17	19.9	41.8	89.4
40	17	20.0	42.3	87.8
AVG		**19**	**37**	**80**
SD		**2**	**4**	**10**
n		**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	Accumulated PFOS intake (ng) in time			
		t=1	t=2	t=3	t=4
1	1	347.9	690.5	1101.4	1487.3
2	1	347.9	690.5	1101.4	1487.3
3	1	347.9	690.5	1101.4	1487.3
4	2	390.1	761.5	1229.1	1672.6
5	2	390.1	761.5	1229.1	1672.6
6	3	314.4	628.8	969.9	1333.3
7	3	314.4	628.8	969.9	1333.3
8	4	452.4	924.5	1472.5	1978.4
9	4	452.4	924.5	1472.5	1978.4
10	4	452.4	924.5	1472.5	1978.4
AVG		**381**	**763**	**1212**	**1641**
SD		**55**	**120**	**199**	**259**
n		**10**	**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	Accumulated PFOS intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
1	1	12.7	24.8	52.2
2	1	12.1	24.1	51.8
3	1	12.0	23.5	49.4
4	2	14.4	27.9	58.7
5	2	16.0	30.3	65.1
6	3	11.6	22.6	49.0
7	3	11.6	22.9	48.0
8	4	15.3	31.0	65.3
9	4	15.0	30.0	63.6
10	4	13.5	27.4	60.0
AVG		**13**	**26**	**56**
SD		**2**	**3**	**7**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Accumulated PFOS intake (ng) in time			
		t=1	t=2	t=3	t=4
11	5	279.9	558.2	891.7	1227.3
12	5	279.9	558.2	891.7	1227.3
13	5	279.9	558.2	891.7	1227.3
14	6	232.3	465.2	735.0	981.2
15	6	232.3	465.2	735.0	981.2
16	7	245.6	525.1	784.0	1090.7
17	7	245.6	525.1	784.0	1090.7
18	8	286.3	569.5	896.5	1236.5
19	8	286.3	569.5	896.5	1236.5
20	8	286.3	569.5	896.5	1236.5
AVG		**265**	**536**	**840**	**1154**
SD		**23**	**41**	**71**	**108**
n		**10**	**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Accumulated PFOS intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
11	5	10.9	21.8	45.8
12	5	10.2	20.0	43.5
13	5	9.2	18.1	38.5
14	6	10.3	20.8	42.8
15	6	8.3	16.2	32.5
16	7	7.6	15.8	31.9
17	7	8.1	17.0	33.0
18	8	9.6	19.2	40.9
19	8	9.3	18.1	40.0
20	8	9.7	19.0	40.8
AVG		**9**	**19**	**39**
SD		**1**	**2**	**5**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Accumulated PFOS intake (ng) in time			
		t=1	t=2	t=3	t=4
21	9	301.9	602.5	942.7	1308.7
22	9	301.9	602.5	942.7	1308.7
23	9	301.9	602.5	942.7	1308.7
24	9	301.9	602.5	942.7	1308.7
25	10	251.2	508.6	807.8	1103.1
26	10	251.2	508.6	807.8	1103.1
27	11B	324.7	613.8	980.1	1355.8
28	11A	289.1	584.4	960.0	1304.8
29	12	286.0	583.6	920.6	1262.3
30	12	286.0	583.6	920.6	1262.3
AVG		**290**	**579**	**917**	**1263**
SD		**23**	**39**	**60**	**88**
n		**10**	**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Accumulated PFOS intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
21	9	11.4	22.8	47.8
22	9	10.7	20.8	43.0
23	9	11.0	21.7	46.7
24	9	11.1	22.0	46.7
25	10	9.4	19.3	41.5
26	10	10.4	21.1	45.4
27	11B	11.2	21.3	44.9
28	11A	10.2	20.4	44.7
29	12	10.7	21.8	44.9
30	12	11.4	22.7	48.0
AVG		**11**	**21**	**45**
SD		**1**	**1**	**2**
n		**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Accumulated PFOS intake (ng) in time			
		t=1	t=2	t=3	t=4
31	13	544.8	1079.5	1705.1	2287.3
32	13	544.8	1079.5	1705.1	2287.3
33	14	524.6	1044.9	1595.5	2225.4
34	14	524.6	1044.9	1595.5	2225.4
35	15	552.0	1128.5	1808.8	2447.3
36	15	552.0	1128.5	1808.8	2447.3
37	16	644.3	1252.5	2084.1	2894.1
38	16	644.3	1252.5	2084.1	2894.1
39	17	592.4	1256.8	1977.5	2688.0
40	17	592.4	1256.8	1977.5	2688.0
AVG		**572**	**1152**	**1834**	**2508**
SD		**45**	**92**	**187**	**264**
n		**10**	**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Accumulated PFOS intake in time per kg mouse (µg/kg)		
		t=1	t=2	t=4
31	13	20.5	40.3	83.5
32	13	19.9	39.0	81.1
33	14	21.2	41.0	88.0
34	14	18.8	36.2	75.4
35	15	18.5	37.5	80.0
36	15	18.6	37.4	78.7
37	16	21.4	40.4	88.8
38	16	24.7	47.8	109.2
39	17	21.9	46.0	98.5
40	17	22.0	46.5	96.7
AVG		**21**	**41**	**88**
SD		**2**	**4**	**11**
n		**10**	**10**	**10**

Appendix V Plasma PFOA and PFOS levels

Group 1: 5% fat diet

Mouse#	Cage#	PFOA conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
1	1	<0.5	<0.5	37.3	96.3	173.0
2	1	<0.5	<0.5	45.0	93.3	226.0
3	1	<0.5	<0.5	43.5	102.3	197.5
4	2	<0.5	<0.5	51.2	95.9	178.0
5	2	<0.5	<0.5	56.7	96.8	161.5
6	3	<0.5	<0.5	39.5	108.5	206.5
7	3	<0.5	0.6	50.7	101.0	222.0
8	4	<0.5	<0.5	35.3	101.2	160.0
9	4	<0.5	0.5	64.7	139.8	192.5
10	4	<0.5	<0.5	36.5	104.7	229.8
AVG		**<0.5**	**<0.6**	**46.0**	**104.0**	**194.7**
SD		**-**	**-**	**9.7**	**13.4**	**26.2**
n		**10**	**10**	**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	PFOA conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
11	5	<0.5	<0.5	28.6	75.3	141.5
12	5	<0.5	0.7	31.3	81.8	154.0
13	5	<0.5	<0.5	31.0	77.9	143.0
14	6	<0.5	<0.5	36.3	85.6	165.5
15	6	<0.5	<0.5	44.8	86.1	159.0
16	7	<0.5	<0.5	42.4	109.5	177.5
17	7	<0.5	<0.5	32.0	68.9	152.5
18	8	<0.5	<0.5	39.0	92.7	155.5
19	8	<0.5	<0.5	33.0	71.7	145.0
20	8	<0.5	0.6	36.4	70.1	180.0
AVG		**<0.5**	**<0.7**	**35.5**	**81.9**	**157.4**
SD		**-**	**-**	**5.3**	**12.4**	**13.5**
n		**10**	**10**	**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	PFOA conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
21	9	<0.5	<0.5	26.5	61.7	124.0
22	9	<0.5	0.5	29.6	60.1	136.8
23	9	<0.5	<0.5	31.4	70.3	157.0
24	9	<0.5	<0.5	28.1	74.5	154.0
25	10	<0.5	0.5	27.3	63.4	144.5
26	10	<0.5	<0.5	34.0	67.2	143.0
27	11B	<0.5	0.5	29.2	56.1	122.5
28	11A	<0.5	0.7	32.1	69.2	154.3
29	12	<0.5	0.5	30.7	67.7	138.0
30	12	<0.5	0.6	30.8	66.0	158.0
AVG		**<0.5**	**<0.7**	**29.9**	**65.6**	**143.2**
SD		**-**	**-**	**2.3**	**5.4**	**13.0**
n		**10**	**10**	**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	PFOA conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
31	13	<0.5	0.7	28.5	65.8	119.5
32	13	<0.5	<0.5	27.6	65.9	129.0
33	14	<0.5	0.8	21.2	60.1	133.0
34	14	<0.5	1	32.3	89.0	134.5
35	15	<0.5	0.6	24.7	56.4	105.5
36	15	<0.5	0.6	23.6	60.8	117.0
37	16	<0.5	0.7	33.5	75.9	145.5
38	16	<0.5	0.8	28.7	61.8	132.0
39	17	<0.5	0.8	35.1	68.7	114.5
40	17	<0.5	0.7	31.6	65.3	152.8
AVG		**<0.5**	**<1.0**	**28.6**	**66.9**	**128.3**
SD		**-**	**-**	**4.5**	**9.4**	**14.5**
n		**10**	**10**	**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	PFOS conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
1	1	<0.5	<0.5	16.0	36.4	68.7
2	1	<0.5	<0.5	12.1	27.9	84.4
3	1	<0.5	<0.5	13.3	157.5	178.0
4	2	<0.5	<0.5	21.3	46.8	87.1
5	2	<0.5	<0.5	21.2	41.7	72.1
6	3	<0.5	<0.5	16.4	46.6	88.5
7	3	<0.5	<0.5	18.4	42.0	109.0
8	4	<0.5	<0.5	18.0	42.9	75.7
9	4	<0.5	<0.5	18.1	58.7	102.1
10	4	<0.5	<0.5	50.1	68.4	110.0
AVG		**<0.5**	**<0.5**	**20.5**	**56.9**	**97.5**
SD		**-**	**-**	**10.8**	**37.1**	**31.8**
n		**10**	**10**	**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	PFOS conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
11	5	<0.5	<0.5	16.4	29.8	96.2
12	5	<0.5	<0.5	23.3	32.1	74.8
13	5	<0.5	<0.5	12.9	28.6	66.8
14	6	<0.5	<0.5	11.5	35.6	82.3
15	6	<0.5	<0.5	11.6	30.3	63.5
16	7	<0.5	<0.5	16.0	40.3	72.0
17	7	<0.5	<0.5	15.1	26.7	55.5
18	8	<0.5	<0.5	20.2	45.6	82.7
19	8	<0.5	<0.5	12.5	29.4	138.0
20	8	<0.5	<0.5	34.2	32.1	77.2
AVG		**<0.5**	**<0.5**	**17.3**	**33.0**	**80.9**
SD		**-**	**-**	**7.0**	**5.9**	**23.0**
n		**10**	**10**	**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	PFOS conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
21	9	<0.5	<0.5	18.2	39.8	69.9
22	9	<0.5	<0.5	11.2	23.0	48.1
23	9	<0.5	<0.5	19.7	28.9	64.0
24	9	<0.5	<0.5	29.2	32.2	71.1
25	10	<0.5	<0.5	10.0	21.0	65.4
26	10	<0.5	<0.5	16.6	29.4	143.3
27	11B	<0.5	<0.5	16.9	28.5	61.1
28	11A	<0.5	<0.5	15.2	23.6	53.3
29	12	<0.5	<0.5	9.9	30.7	66.1
30	12	<0.5	<0.5	10.6	19.2	53.5
AVG		**<0.5**	**<0.5**	**15.7**	**27.6**	**69.6**
SD		**-**	**-**	**6.0**	**6.1**	**27.0**
n		**10**	**10**	**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	PFOS conc in plasma (ng/mL)				
		t=-4	t=0	t=1	t=2	t=4
31	13	<0.5	<0.5	12.9	30.8	58.2
32	13	<0.5	<0.5	12.0	43.4	84.7
33	14	<0.5	<0.5	8.2	24.8	70.8
34	14	<0.5	<0.5	22.0	41.2	69.2
35	15	<0.5	<0.5	25.1	32.3	64.0
36	15	<0.5	<0.5	10.5	34.8	83.5
37	16	<0.5	<0.5	19.7	37.8	87.2
38	16	<0.5	<0.5	15.0	36.2	75.7
39	17	<0.5	<0.5	23.2	70.5	89.9
40	17	<0.5	<0.5	16.6	58.9	98.0
AVG		**<0.5**	**<0.5**	**16.5**	**41.1**	**78.1**
SD		**-**	**-**	**5.8**	**13.8**	**12.6**
n		**10**	**10**	**10**	**10**	**10**

Appendix VI PFOA and PFOS dose as percentage of intake

Group 1: 5% fat diet

Mouse#	Cage#	Estimated PFOA dose in body (µg/kg)		
		t=1	t=2	t=4
1	1	7.5	19.3	34.6
2	1	9.0	18.7	45.2
3	1	8.7	20.5	39.5
4	2	10.2	19.2	35.6
5	2	11.3	19.4	32.3
6	3	7.9	21.7	41.3
7	3	10.1	20.2	44.4
8	4	7.1	20.2	32.0
9	4	12.9	28.0	38.5
10	4	7.3	20.9	46.0
AVG		**9.2**	**20.8**	**38.9**
SD		**1.9**	**2.7**	**5.2**
n		**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	PFOA dose in body (% of PFOA intake)		
		t=1	t=2	t=4
1	1	37%	49%	42%
2	1	46%	48%	55%
3	1	45%	55%	50%
4	2	45%	43%	38%
5	2	45%	40%	31%
6	3	43%	60%	53%
7	3	55%	55%	58%
8	4	29%	41%	31%
9	4	54%	58%	38%
10	4	34%	48%	48%
AVG		**43%**	**50%**	**44%**
SD		**8%**	**7%**	**10%**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Estimated PFOA dose in body (µg/kg)		
		t=1	t=2	t=4
11	5	5.7	15.1	28.3
12	5	6.3	16.4	30.8
13	5	6.2	15.6	28.6
14	6	7.3	17.1	33.1
15	6	9.0	17.2	31.8
16	7	8.5	21.9	35.5
17	7	6.4	13.8	30.5
18	8	7.8	18.5	31.1
19	8	6.6	14.3	29.0
20	8	7.3	14.0	36.0
AVG		**7.1**	**16.4**	**31.5**
SD		**1.1**	**2.5**	**2.7**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	PFOA dose in body (% of PFOA intake)		
		t=1	t=2	t=4
11	5	23%	31%	27%
12	5	27%	36%	31%
13	5	30%	38%	33%
14	6	31%	36%	34%
15	6	48%	47%	43%
16	7	49%	61%	49%
17	7	35%	36%	41%
18	8	36%	43%	34%
19	8	31%	35%	32%
20	8	33%	33%	39%
AVG		**34%**	**40%**	**36%**
SD		**8%**	**9%**	**7%**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Estimated PFOA dose in body (µg/kg)		
		t=1	t=2	t=4
21	9	5.3	12.3	24.8
22	9	5.9	12.0	27.4
23	9	6.3	14.1	31.4
24	9	5.6	14.9	30.8
25	10	5.5	12.7	28.9
26	10	6.8	13.4	28.6
27	11B	5.8	11.2	24.5
28	11A	6.4	13.8	30.9
29	12	6.1	13.5	27.6
30	12	6.2	13.2	31.6
AVG		**6.0**	**13.1**	**28.6**
SD		**0.5**	**1.1**	**2.6**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	PFOA dose in body (% of PFOA intake)		
		t=1	t=2	t=4
21	9	28%	33%	32%
22	9	34%	35%	39%
23	9	35%	40%	41%
24	9	31%	41%	40%
25	10	35%	40%	43%
26	10	40%	39%	38%
27	11B	32%	32%	33%
28	11A	38%	41%	42%
29	12	35%	38%	38%
30	12	33%	35%	40%
AVG		**34%**	**38%**	**39%**
SD		**3%**	**3%**	**4%**
n		**10**	**10**	**10**

Estimated PFOA or PFOS dose in body (ng/kg)= 200x PFOA or PFOS dose in plasma (ng/mL)
(Estimation based on internal data from the Sponsor)

Group 4: 35% fat diet

Mouse#	Cage#	Estimated PFOA dose in body (µg/kg)		
		t=1	t=2	t=4
31	13	5.7	13.2	23.9
32	13	5.5	13.2	25.8
33	14	4.2	12.0	26.6
34	14	6.5	17.8	26.9
35	15	4.9	11.3	21.1
36	15	4.7	12.2	23.4
37	16	6.7	15.2	29.1
38	16	5.7	12.4	26.4
39	17	7.0	13.7	22.9
40	17	6.3	13.1	30.6
AVG		**5.7**	**13.4**	**25.7**
SD		**0.9**	**1.9**	**2.9**
n		**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	PFOA dose in body (% of PFOA intake)		
		t=1	t=2	t=4
31	13	31%	36%	32%
32	13	30%	37%	35%
33	14	22%	32%	33%
34	14	38%	54%	39%
35	15	29%	33%	29%
36	15	28%	36%	33%
37	16	34%	41%	36%
38	16	26%	28%	27%
39	17	35%	33%	26%
40	17	32%	31%	35%
AVG		**31%**	**36%**	**32%**
SD		**5%**	**7%**	**4%**
n		**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (µg/kg)		
		t=1	t=2	t=4
1	1	3.2	7.3	13.7
2	1	2.4	5.6	16.9
3	1	2.7	31.5	35.6
4	2	4.3	9.4	17.4
5	2	4.2	8.3	14.4
6	3	3.3	9.3	17.7
7	3	3.7	8.4	21.8
8	4	3.6	8.6	15.1
9	4	3.6	11.7	20.4
10	4	10.0	13.7	22.0
AVG		**4.1**	**11.4**	**19.5**
SD		**2.2**	**7.4**	**6.4**
n		**10**	**10**	**10**

Group 1: 5% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (% of PFOS intake)		
		t=1	t=2	t=4
1	1	25.2%	29.3%	26.3%
2	1	19.8%	23.1%	32.6%
3	1	22.2%	134.1%	72.0%
4	2	29.6%	33.5%	29.7%
5	2	26.5%	27.5%	22.2%
6	3	28.2%	41.2%	36.1%
7	3	31.6%	36.6%	45.5%
8	4	23.4%	27.7%	23.2%
9	4	24.2%	39.1%	32.1%
10	4	74.3%	50.0%	36.7%
AVG		**30.5%**	**44.2%**	**35.6%**
SD		**15.8%**	**32.6%**	**14.5%**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (µg/kg)		
		t=1	t=2	t=4
11	5	3.3	6.0	19.2
12	5	4.7	6.4	15.0
13	5	2.6	5.7	13.4
14	6	2.3	7.1	16.5
15	6	2.3	6.1	12.7
16	7	3.2	8.1	14.4
17	7	3.0	5.3	11.1
18	8	4.0	9.1	16.5
19	8	2.5	5.9	27.6
20	8	6.8	6.4	15.4
AVG		**3.5**	**6.6**	**16.2**
SD		**1.4**	**1.2**	**4.6**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (% of PFOS intake)		
		t=1	t=2	t=4
11	5	30.0%	27.3%	42.0%
12	5	45.9%	32.0%	34.4%
13	5	28.0%	31.6%	34.7%
14	6	22.1%	34.2%	38.4%
15	6	27.9%	37.5%	39.1%
16	7	42.1%	50.9%	45.1%
17	7	37.3%	31.3%	33.7%
18	8	41.8%	47.4%	40.4%
19	8	26.8%	32.4%	69.0%
20	8	70.4%	33.8%	37.8%
AVG		**37.2%**	**35.8%**	**41.5%**
SD		**14.1%**	**7.5%**	**10.3%**
n		**10**	**10**	**10**

Estimated PFOA or PFOS dose in body (ng/kg)= 200x PFOA or PFOS dose in plasma (ng/mL)

Group 3: 25% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (µg/kg)		
		t=1	t=2	t=4
21	9	3.6	8.0	14.0
22	9	2.2	4.6	9.6
23	9	3.9	5.8	12.8
24	9	5.8	6.4	14.2
25	10	2.0	4.2	13.1
26	10	3.3	5.9	28.7
27	11	3.4	5.7	12.2
28	11	3.0	4.7	10.7
29	12	2.0	6.1	13.2
30	12	2.1	3.8	10.7
AVG		**3.1**	**5.5**	**13.9**
SD		**1.2**	**1.2**	**5.4**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (% of PFOS intake)		
		t=1	t=2	t=4
21	9	31.7%	34.9%	29.3%
22	9	20.8%	22.0%	22.3%
23	9	35.7%	26.7%	27.4%
24	9	52.9%	29.3%	30.4%
25	10	21.2%	21.8%	31.5%
26	10	32.0%	27.8%	63.1%
27	11B	30.1%	26.7%	27.2%
28	11A	29.8%	23.2%	23.9%
29	12	18.5%	28.2%	29.4%
30	12	18.5%	16.9%	22.3%
AVG		**29.1%**	**25.7%**	**30.7%**
SD		**10.4%**	**5.0%**	**11.9%**
n		**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (µg/kg)		
		t=1	t=2	t=4
31	13	2.6	6.2	11.6
32	13	2.4	8.7	16.9
33	14	1.6	5.0	14.2
34	14	4.4	8.2	13.8
35	15	5.0	6.5	12.8
36	15	2.1	7.0	16.7
37	16	3.9	7.6	17.4
38	16	3.0	7.2	15.1
39	17	4.6	14.1	18.0
40	17	3.3	11.8	19.6
AVG		**3.3**	**8.2**	**15.6**
SD		**1.2**	**2.8**	**2.5**
n		**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Estimated PFOS dose in body (% of PFOS intake)		
		t=1	t=2	t=4
31	13	12.6%	15.3%	13.9%
32	13	12.0%	22.3%	20.9%
33	14	7.8%	12.1%	16.1%
34	14	23.4%	22.8%	18.3%
35	15	27.1%	17.2%	16.0%
36	15	11.2%	18.6%	21.2%
37	16	18.4%	18.7%	19.6%
38	16	12.1%	15.1%	13.9%
39	17	21.1%	30.6%	18.3%
40	17	15.0%	25.3%	20.3%
AVG		**16.1%**	**19.8%**	**17.8%**
SD		**6.2%**	**5.5%**	**2.7%**
n		**10**	**10**	**10**

Estimated PFOA or PFOS dose in body (ng/kg)= 200x PFOA or PFOS dose in plasma (ng/mL)

Appendix VII Plasma total cholesterol

Group 1: 5% fat diet

Mouse#	Cage#	cholesterol (mM) in time			
		t=-4	t=0	t=2	t=4
1	1	2.43	11.35	13.74	13.92
2	1	2.40	14.46	17.58	14.00
3	1	2.60	10.42	9.01	10.42
4	2	2.60	9.62	12.08	11.86
5	2	2.47	9.33	14.56	12.15
6	3	2.71	16.43	22.71	16.77
7	3	3.20	13.16	16.15	12.82
8	4	2.95	16.55	16.26	14.58
9	4	1.49	5.41	4.66	12.03
10	4	2.47	12.85	12.91	5.14
AVG		**2.5**	**12.0**	**14.0**	**12.4**
SD		**0.4**	**3.5**	**4.9**	**3.1**
n		**10**	**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	cholesterol (mM) in time			
		t=-4	t=0	t=2	t=4
11	5	3.20	6.85	7.80	5.79
12	5	3.11	6.46	7.13	5.61
13	5	2.90	10.46	13.00	9.03
14	6	2.08	10.39	9.10	7.49
15	6	1.88	5.40	6.76	5.06
16	7	2.00	3.62	2.79	3.18
17	7	2.73	9.52	8.96	8.04
18	8	2.13	7.47	7.41	7.46
19	8	1.72	8.39	9.96	10.97
20	8	2.78	6.95	13.07	5.24
AVG		**2.5**	**7.5**	**8.6**	**6.8**
SD		**0.5**	**2.2**	**3.0**	**2.3**
n		**10**	**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	cholesterol (mM) in time			
		t=-4	t=0	t=2	t=4
21	9	2.32	5.09	3.99	4.53
22	9	2.65	5.98	5.37	6.11
23	9	2.05	7.40	5.51	5.30
24	9	2.18	5.91	4.48	4.51
25	10	2.12	6.46	6.39	5.66
26	10	2.35	4.27	5.01	5.95
27	11	2.78	4.27	5.79	5.85
28	11	3.53	5.94	8.04	5.85
29	12	2.23	4.59	5.07	5.69
30	12	1.95	5.34	4.78	6.26
AVG		**2.4**	**5.5**	**5.4**	**5.6**
SD		**0.5**	**1.0**	**1.1**	**0.6**
n		**10**	**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	cholesterol (mM) in time			
		t=-4	t=0	t=2	t=4
31	13	2.50	4.63	4.57	4.22
32	13	3.31	5.16	5.24	5.26
33	14	2.33	4.39	4.70	4.74
34	14	2.55	4.30	4.92	5.09
35	15	3.21	4.37	4.59	4.38
36	15	2.62	4.22	4.63	4.74
37	16	1.64	7.23	6.23	6.41
38	16	2.17	4.40	4.10	3.51
39	17	2.55	6.01	5.19	4.90
40	17	2.70	4.32	4.71	4.46
AVG		**2.6**	**4.9**	**4.9**	**4.8**
SD		**0.5**	**1.0**	**0.6**	**0.8**
n		**10**	**10**	**10**	**10**

Appendix VIII Plasma triglycerides

Group 1: 5% fat diet

Mouse#	Cage#	triglycerides (mM) in time			
		t=-4	t=0	t=2	t=4
1	1	3.78	4.92	3.57	2.91
2	1	4.42	5.58	4.84	2.41
3	1	4.43	3.96	3.32	3.03
4	2	3.59	3.54	3.97	3.88
5	2	3.53	2.89	3.65	2.93
6	3	3.56	4.55	3.44	3.73
7	3	4.23	3.38	4.25	3.22
8	4	3.44	4.48	3.41	3.16
9	4	0.91	1.58	1.36	3.23
10	4	3.48	4.23	4.15	1.46
AVG		**3.5**	**3.9**	**3.6**	**3.0**
SD		**1.0**	**1.1**	**0.9**	**0.7**
n		**10**	**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	triglycerides (mM) in time			
		t=-4	t=0	t=2	t=4
11	5	3.98	3.62	1.97	3.30
12	5	4.83	3.18	3.35	3.62
13	5	4.26	4.23	4.97	3.91
14	6	2.66	4.78	3.95	3.38
15	6	3.14	3.67	3.20	2.25
16	7	1.09	1.70	1.72	1.42
17	7	4.63	4.57	2.97	3.13
18	8	3.64	3.60	3.57	2.97
19	8	2.54	4.41	3.65	4.60
20	8	4.78	3.66	0.58	2.47
AVG		**3.6**	**3.7**	**3.0**	**3.1**
SD		**1.2**	**0.9**	**1.3**	**0.9**
n		**10**	**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	triglycerides (mM) in time			
		t=-4	t=0	t=2	t=4
21	9	3.81	2.21	1.35	2.11
22	9	4.03	4.09	1.83	2.70
23	9	3.05	4.47	2.48	2.36
24	9	2.90	2.86	1.97	1.81
25	10	3.22	2.44	1.58	1.59
26	10	2.85	1.24	1.16	1.20
27	11	4.75	1.04	0.70	1.08
28	11	5.58	2.48	0.81	1.70
29	12	3.22	2.34	1.72	2.12
30	12	3.39	1.92	1.59	1.96
AVG		**3.7**	**2.5**	**1.5**	**1.9**
SD		**0.9**	**1.1**	**0.5**	**0.5**
n		**10**	**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	triglycerides (mM) in time			
		t=-4	t=0	t=2	t=4
31	13	2.72	1.82	1.22	1.29
32	13	4.72	2.15	1.64	1.24
33	14	3.21	1.67	1.06	1.39
34	14	3.98	2.00	1.36	1.38
35	15	4.29	1.47	1.38	0.90
36	15	4.19	1.65	1.52	1.25
37	16	2.68	2.23	2.41	2.19
38	16	3.48	1.87	1.13	1.10
39	17	3.36	2.91	1.33	1.52
40	17	3.47	1.42	1.03	1.40
AVG		**3.6**	**1.9**	**1.4**	**1.4**
SD		**0.7**	**0.4**	**0.4**	**0.3**
n		**10**	**10**	**10**	**10**

Appendix IX Plasma HDL-cholesterol

Group 1: 5% fat diet

Mouse#	Cage#	HDL-cholesterol (mM) in time		
		t=0	t=2	t=4
1	1	0.51	0.41	0.74
2	1	0.60	0.50	0.81
3	1	0.45	0.41	0.56
4	2	0.53	0.32	0.34
5	2	0.24	0.61	0.24
6	3	0.46	0.68	0.73
7	3	0.78	0.62	0.78
8	4	0.23	0.27	0.41
9	4	0.28	0.24	0.51
10	4	0.21	0.24	0.23
AVG		**0.43**	**0.43**	**0.54**
SD		**0.19**	**0.16**	**0.22**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	HDL-cholesterol (mM) in time		
		t=0	t=2	t=4
11	5	0.95	1.20	0.78
12	5	0.85	0.67	0.63
13	5	0.55	0.59	0.61
14	6	0.56	0.88	0.73
15	6	0.50	0.58	0.88
16	7	0.26	0.26	0.33
17	7	0.82	0.61	0.86
18	8	0.75	0.67	0.63
19	8	0.34	0.27	0.43
20	8	0.68	0.91	0.81
AVG		**0.63**	**0.66**	**0.67**
SD		**0.22**	**0.28**	**0.18**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	HDL-cholesterol (mM) in time		
		t=0	t=2	t=4
21	9	1.19	1.11	1.01
22	9	0.92	1.30	1.26
23	9	0.61	0.47	0.90
24	9	0.88	0.73	0.96
25	10	1.03	1.42	1.36
26	10	0.88	1.14	1.77
27	11	1.12	2.00	1.48
28	11	0.85	1.21	1.50
29	12	0.83	1.01	1.18
30	12	0.83	0.80	1.18
AVG		**0.91**	**1.12**	**1.26**
SD		**0.16**	**0.42**	**0.27**
n		**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	HDL-cholesterol (mM) in time		
		t=0	t=2	t=4
31	13	1.39	1.24	1.21
32	13	1.02	1.45	1.46
33	14	1.03	0.47	1.43
34	14	0.88	1.36	1.50
35	15	1.12	1.11	1.41
36	15	1.52	1.77	1.65
37	16	0.48	0.89	0.61
38	16	1.86	1.23	1.13
39	17	0.48	0.86	1.20
40	17	0.80	1.24	1.26
AVG		**1.06**	**1.16**	**1.29**
SD		**0.44**	**0.36**	**0.29**
n		**10**	**10**	**10**

Appendix X Lipoprotein profiles

Lipoprotein profiles t=-4 weeks

Fraction	Cholesterol (mM)			
	Group 1	Group 2	Group 3	Group 4
1	0.00	0.02	0.00	0.00
2	0.00	0.01	0.00	0.00
3	0.01	0.02	0.01	0.00
4	0.10	0.15	0.14	0.14
5	0.21	0.22	0.21	0.20
6	0.15	0.15	0.16	0.13
7	0.11	0.12	0.11	0.11
8	0.12	0.14	0.11	0.12
9	0.14	0.12	0.09	0.11
10	0.14	0.15	0.12	0.13
11	0.12	0.12	0.11	0.12
12	0.12	0.13	0.12	0.11
13	0.10	0.11	0.09	0.09
14	0.07	0.07	0.07	0.05
15	0.05	0.05	0.04	0.03
16	0.02	0.03	0.01	0.01
17	0.02	0.03	0.00	0.01
18	0.03	0.04	0.02	0.02
19	0.07	0.07	0.05	0.05
20	0.11	0.09	0.07	0.08
21	0.10	0.10	0.07	0.07
22	0.07	0.05	0.04	0.04
23	0.04	0.04	0.01	0.01
24	0.02	0.03	0.00	0.00
Sum	1.9	2.1	1.6	1.6
TC	2.5	2.5	2.4	2.6
% Recovery	76%	84%	68%	64%

Fraction	Phospholipids (mg/dL)			
	Group 1	Group 2	Group 3	Group 4
1	0.22	0.72	0.37	0.37
2	0.47	0.72	0.62	0.37
3	0.22	0.47	0.62	0.37
4	2.96	4.45	4.08	3.84
5	6.44	6.44	6.07	6.81
6	4.70	4.70	4.83	4.08
7	3.45	3.70	3.34	3.34
8	3.45	3.95	3.34	3.84
9	3.70	3.70	3.09	3.34
10	4.45	4.45	3.84	4.08
11	3.45	3.70	3.34	3.59
12	3.45	3.70	3.59	3.09
13	2.71	3.45	2.35	2.60
14	2.46	1.96	1.85	1.85
15	1.22	1.71	1.11	1.11
16	0.72	0.97	0.86	1.11
17	0.97	1.22	0.62	0.62
18	1.46	1.71	1.11	1.36
19	1.96	2.71	2.10	2.60
20	3.95	3.45	3.34	3.84
21	3.70	3.95	2.85	3.59
22	2.46	2.21	2.10	2.35
23	1.46	1.46	1.11	1.36
24	0.97	1.22	0.86	1.11

Lipoprotein profiles t=0 weeks

Fraction	Cholesterol (mM)			
	Group 1	Group 2	Group 3	Group 4
1	0.00	0.02	0.008	0.020
2	0.00	0.02	0.014	0.020
3	0.05	0.07	0.04	0.046
4	1.69	0.86	0.364	0.258
5	2.59	1.45	0.562	0.326
6	1.64	0.92	0.394	0.226
7	1.02	0.62	0.3	0.188
8	0.72	0.47	0.252	0.188
9	0.66	0.43	0.258	0.208
10	0.54	0.41	0.294	0.244
11	0.48	0.42	0.314	0.258
12	0.42	0.33	0.294	0.282
13	0.31	0.25	0.232	0.244
14	0.21	0.17	0.176	0.170
15	0.15	0.12	0.12	0.126
16	0.09	0.07	0.102	0.108
17	0.10	0.09	0.114	0.140
18	0.09	0.12	0.188	0.232
19	0.17	0.21	0.364	0.468
20	0.21	0.28	0.456	0.544
21	0.17	0.24	0.37	0.376
22	0.12	0.13	0.182	0.226
23	0.06	0.07	0.102	0.102
24	0.04	0.03	0.058	0.064
Sum	11.52	7.8	5.6	5.1
TC	12.0	7.5	5.5	4.9
% Recovery	96%	103%	101%	103%

Fraction	Phospholipids (mg/dL)			
	Group 1	Group 2	Group 3	Group 4
1	0.00	0.10	0.00	0.00
2	0.10	0.10	0.00	0.00
3	0.34	0.59	0.22	0.22
4	17.35	11.19	5.39	4.90
5	28.94	21.05	9.33	6.38
6	20.31	14.15	7.12	4.41
7	12.67	9.46	5.15	3.42
8	9.46	8.23	4.41	3.67
9	9.71	7.74	5.15	4.16
10	8.48	7.74	6.13	5.15
11	7.49	8.23	6.13	5.15
12	6.75	6.26	5.64	6.13
13	4.78	4.54	4.41	4.90
14	3.06	3.55	3.18	2.93
15	2.32	2.07	2.19	2.68
16	1.58	1.58	2.44	2.68
17	1.58	1.82	2.68	4.16
18	2.32	3.80	6.38	9.09
19	5.03	7.74	14.01	19.68
20	7.74	11.68	18.94	24.61
21	6.51	10.70	15.49	17.22
22	4.54	5.52	7.61	11.30
23	2.32	3.55	4.65	4.65
24	1.82	1.60	1.94	2.19

Lipoprotein profiles t=2 weeks

Fraction	Cholesterol (mM)			
	Group 1	Group 2	Group 3	Group 4
1	0.00	0.00	0.006	0.018
2	0.01	0.00	0.012	0.006
3	0.14	0.07	0.054	0.030
4	2.19	0.86	0.182	0.118
5	2.74	1.27	0.314	0.186
6	1.75	0.70	0.192	0.122
7	0.98	0.46	0.152	0.094
8	0.80	0.39	0.152	0.118
9	0.64	0.37	0.164	0.122
10	0.60	0.34	0.198	0.164
11	0.48	0.36	0.234	0.192
12	0.38	0.26	0.228	0.234
13	0.28	0.23	0.192	0.182
14	0.16	0.14	0.146	0.152
15	0.10	0.08	0.122	0.128
16	0.07	0.06	0.128	0.140
17	0.07	0.06	0.164	0.198
18	0.07	0.14	0.304	0.344
19	0.14	0.26	0.548	0.652
20	0.16	0.32	0.506	0.646
21	0.11	0.23	0.35	0.430
22	0.06	0.10	0.176	0.222
23	0.04	0.06	0.088	0.112
24	0.01	0.01	0.06	0.076
Sum	11.98	6.8	4.7	4.7
TC	14.0	8.6	5.4	4.9
% Recovery	86%	79%	86%	96%

Fraction	Phospholipids (mg/dL)			
	Group 1	Group 2	Group 3	Group 4
1	0.18	0.43	0.33	0.58
2	0.43	0.43	0.33	0.08
3	1.67	0.92	0.82	0.58
4	21.59	9.89	2.07	2.07
5	29.80	15.61	4.82	3.57
6	19.84	9.39	3.07	2.32
7	12.13	6.40	2.57	1.82
8	10.13	6.15	2.32	2.07
9	8.89	6.15	2.82	2.57
10	9.39	6.15	3.32	3.32
11	7.40	6.40	4.07	4.07
12	5.90	4.91	4.07	4.57
13	4.16	4.16	3.32	3.82
14	2.91	2.42	2.57	3.07
15	1.67	1.67	2.32	2.82
16	1.42	1.42	2.32	3.07
17	1.42	1.67	4.07	5.31
18	2.42	4.66	8.81	10.30
19	5.65	9.39	16.29	21.03
20	6.40	10.88	15.29	20.03
21	4.66	8.39	11.05	14.54
22	2.91	4.16	5.56	7.81
23	1.92	2.67	3.07	3.82
24	1.42	1.67	2.07	3.57

Lipoprotein profiles t=4 weeks

Fraction	Cholesterol (mM)			
	Group 1	Group 2	Group 3	Group 4
1	0.00	0.01	0.006	0.018
2	0.01	0.01	0.012	0.012
3	0.09	0.06	0.048	0.036
4	1.75	0.70	0.262	0.178
5	2.31	1.09	0.412	0.238
6	1.52	0.65	0.292	0.154
7	0.91	0.42	0.226	0.126
8	0.65	0.33	0.19	0.142
9	0.56	0.31	0.202	0.154
10	0.53	0.31	0.244	0.184
11	0.42	0.32	0.268	0.208
12	0.37	0.24	0.244	0.238
13	0.28	0.18	0.214	0.196
14	0.18	0.12	0.16	0.154
15	0.12	0.08	0.12	0.114
16	0.07	0.05	0.108	0.114
17	0.07	0.06	0.136	0.154
18	0.07	0.10	0.244	0.268
19	0.13	0.19	0.424	0.524
20	0.16	0.25	0.482	0.530
21	0.13	0.18	0.346	0.358
22	0.08	0.09	0.166	0.208
23	0.04	0.05	0.096	0.096
24	0.02	0.02	0.06	0.066
Sum	10.48	5.8	5.0	4.5
TC	12.4	6.8	5.6	4.8
% Recovery	85%	85%	89%	94%

Fraction	Phospholipids (mg/dL)			
	Group 1	Group 2	Group 3	Group 4
1	0.07	0.31	0.06	0.31
2	0.31	0.31	0.06	0.06
3	0.80	0.56	0.56	0.31
4	17.27	7.93	3.52	3.27
5	26.61	14.57	6.73	4.76
6	18.75	9.65	4.76	3.03
7	11.37	6.21	3.77	2.53
8	8.42	5.72	3.27	2.53
9	8.67	5.72	3.77	3.03
10	8.42	5.72	4.51	3.77
11	6.46	5.47	5.00	4.02
12	5.47	4.25	4.76	5.00
13	4.00	3.26	3.77	4.02
14	2.77	2.53	2.78	2.78
15	1.79	1.54	2.29	2.53
16	1.30	1.05	2.29	2.78
17	1.30	1.30	3.27	4.51
18	2.03	3.26	7.23	8.71
19	4.74	6.70	14.89	18.10
20	6.70	9.16	15.88	19.83
21	5.23	7.44	12.42	15.14
22	3.26	4.00	6.49	8.46
23	2.03	2.53	3.77	4.02
24	1.54	1.30	1.79	2.53

Appendix XI Average plasma cholesterol levels and relative PFOA/PFOS doses

Group 1: 5% fat diet

Mouse#	Cage#	Average Cholesterol (mM) t=0-4 weeks	Average relative PFOA dose (%) t=1-4 weeks	Average relative PFOS dose (%) t=1-4 weeks
1	1	13.00	42%	27%
2	1	15.35	50%	25%
3	1	9.95	50%	76%
4	2	11.19	42%	31%
5	2	12.02	39%	25%
6	3	18.63	52%	35%
7	3	14.04	56%	38%
8	4	15.80	33%	25%
9	4	7.37	50%	32%
10	4	10.30	43%	54%
AVG		**12.8**	**46%**	**37%**
SD		**3.3**	**7%**	**16%**
n		**10**	**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Average Cholesterol (mM) t=0-4 weeks	Average relative PFOA dose (%) t=1-4 weeks	Average relative PFOS dose (%) t=1-4 weeks
11	5	6.81	27%	33%
12	5	6.40	32%	37%
13	5	10.83	34%	31%
14	6	8.99	34%	32%
15	6	5.74	46%	35%
16	7	3.20	53%	46%
17	7	8.84	37%	34%
18	8	7.44	37%	43%
19	8	9.78	33%	43%
20	8	8.42	35%	47%
AVG		**7.6**	**37%**	**38%**
SD		**2.2**	**8%**	**6%**
n		**10**	**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Average Cholesterol (mM) t=0-4 weeks	Average relative PFOA dose (%) t=1-4 weeks	Average relative PFOS dose (%) t=1-4 weeks
21	9	4.53	31%	32%
22	9	5.82	36%	22%
23	9	6.07	38%	30%
24	9	4.97	38%	38%
25	10	6.17	39%	25%
26	10	5.08	39%	41%
27	11	5.30	32%	28%
28	11	6.61	41%	26%
29	12	5.12	37%	25%
30	12	5.46	36%	19%
AVG		**5.5**	**37%**	**29%**
SD		**0.6**	**3%**	**7%**
n		**10**	**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Average Cholesterol (mM) t=0-4 weeks	Average relative PFOA dose (%) t=0-4 weeks	Average relative PFOS dose (%) t=1-4 weeks
31	13	4.47	33%	14%
32	13	5.22	34%	18%
33	14	4.61	29%	12%
34	14	4.77	44%	22%
35	15	4.45	31%	20%
36	15	4.53	32%	17%
37	16	6.62	37%	19%
38	16	4.00	27%	14%
39	17	5.37	31%	23%
40	17	4.50	32%	20%
AVG		**4.9**	**33%**	**18%**
SD		**0.7**	**5%**	**4%**
n		**10**	**10**	**10**

Appendix XII PFOA and PFOS liver levels

Absolute PFOA and PFOS liver levels

Group 1: 5% fat diet + PFOA/PFOS

Mouse#	Cage#	PFOA liver levels		PFOS liver levels	
		ng/g liver	ng per liver	ng/g liver	ng per liver
1	1	386	660	394	674
2	1	237	493	216	449
3	1	459	973	978	2073
4	2	492	886	773	1391
5	2	435	766	664	1169
6	3	457	754	500	825
7	3	461	784	564	959
8	4	447	769	596	1025
9	4	174	214	231	284
10	4	450	842	612	1144
AVG		**400**	**714**	**553**	**999**
SD		**107**	**218**	**234**	**507**
n		**10**	**10**	**10**	**10**

Group 2: 15% fat diet + PFOA/PFOS

Mouse#	Cage#	PFOA liver levels		PFOS liver levels	
		ng/g liver	ng per liver	ng/g liver	ng per liver
11	5	378	510	534	721
12	5	349	586	379	637
13	5	375	713	414	787
14	6	409	479	472	552
15	6	244	417	201	344
16	7	293	483	324	535
17	7	431	806	392	733
18	8	413	653	527	833
19	8	437	734	956	1606
20	8	494	726	579	851
AVG		**382**	**611**	**478**	**760**
SD		**73**	**134**	**202**	**336**
n		**10**	**10**	**10**	**10**

Group 3: 25% fat diet + PFOA/PFOS

Mouse#	Cage#	PFOA liver levels		PFOS liver levels	
		ng/g liver	ng per liver	ng/g liver	ng per liver
21	9	351	470	388	520
22	9	404	614	304	462
23	9	395	569	398	573
24	9	334	461	366	505
25	10	334	458	332	455
26	10	284	366	591	762
27	11B	402	611	414	629
28	11A	374	561	291	437
29	12	309	392	294	373
30	12	326	378	245	284
AVG		**351**	**488**	**362**	**500**
SD		**41**	**95**	**97**	**134**
n		**10**	**10**	**10**	**10**

Group 4: 35% fat diet + PFOA/PFOS

Mouse#	Cage#	PFOA liver levels		PFOS liver levels	
		ng/g liver	ng per liver	ng/g liver	ng per liver
31	13	353	477	371	501
32	13	333	440	469	619
33	14	397	464	453	530
34	14	337	431	347	444
35	15	378	548	476	690
36	15	368	563	495	757
37	16	266	362	382	520
38	16	351	442	449	566
39	17	411	530	652	841
40	17	339	458	453	612
AVG		**353**	**471**	**455**	**608**
SD		**40**	**61**	**85**	**124**
n		**10**	**10**	**10**	**10**

PFOA and PFOS liver levels as % of food intake

Group 1: 5% fat diet

Mouse#	Cage#	Liver PFOA/PFOS (% of PFOA/PFOS intake)	
		PFOA	PFOS
1	1	28%	45%
2	1	21%	30%
3	1	41%	139%
4	2	33%	83%
5	2	29%	70%
6	3	35%	62%
7	3	37%	72%
8	4	24%	52%
9	4	7%	14%
10	4	27%	58%
AVG		**28%**	**63%**
SD		**10%**	**34%**
n		**10**	**10**

Group 2: 15% fat diet

Mouse#	Cage#	Liver PFOA/PFOS (% of PFOA/PFOS intake)	
		PFOA	PFOS
11	5	18%	59%
12	5	21%	52%
13	5	26%	64%
14	6	22%	56%
15	6	19%	35%
16	7	20%	49%
17	7	33%	67%
18	8	23%	67%
19	8	26%	130%
20	8	26%	69%
AVG		**23%**	**65%**
SD		**4%**	**25%**
n		**10**	**10**

Group 3: 25% fat diet

Mouse#	Cage#	Liver PFOA/PFOS (% of PFOA/PFOS intake)	
		PFOA	PFOS
21	9	22%	40%
22	9	29%	35%
23	9	27%	44%
24	9	21%	39%
25	10	25%	41%
26	10	20%	69%
27	11B	28%	46%
28	11A	26%	33%
29	12	19%	30%
30	12	18%	23%
AVG		**24%**	**40%**
SD		**4%**	**12%**
n		**10**	**10**

Group 4: 35% fat diet

Mouse#	Cage#	Liver PFOA/PFOS (% of PFOA/PFOS intake)	
		PFOA	PFOS
31	13	23%	22%
32	13	21%	27%
33	14	23%	24%
34	14	21%	20%
35	15	25%	28%
36	15	25%	31%
37	16	14%	18%
38	16	17%	20%
39	17	22%	31%
40	17	19%	23%
AVG		**21%**	**24%**
SD		**4%**	**5%**
n		**10**	**10**

Appendix XIII Report on statistical analysis of associations among effects resulting from feeding fat, PFOA, and PFOS in the diet of mice for four weeks by David W. Gaylor, Ph.D.

10/17/11

Statistical Analysis of Associations among Effects Resulting from feeding Fat, PFOA, and PFOS in the diet of Mice for Four Weeks

David W. Gaylor, Ph.D.

Background

A study was conducted at TNO Biosciences in which male mice were fed 5%, 15%, 25%, or 35% fat in the diet for four weeks plus approximately 0.03 ppm of PFOA and PFOS in the diet. PFOA and PFOS concentrations in plasma, HDL and non-HDL cholesterol, and triglycerides were measured on 10 mice per dietary fat group (40 mice total) after various periods of exposure. Several effects due to dietary fat were noted in the TNO study.

The purpose of the following statistical analysis is to exam associations among the endpoints (PFOA and PFOS plasma concentration, HDL and non-HDL cholesterol, and triglycerides) apart from correlations due to dietary fat.

Approach

In this analysis, the results after four weeks of exposure were used. Since dietary fat intake affected several endpoints, the calculation of correlation coefficients between endpoints across the 40 mice is not appropriate. For example, if dietary fat affects two endpoints a correlation between the two endpoints will necessarily occur and it is not possible to determine from the overall correlation coefficient if the two endpoints are associated in addition to the effects due to dietary fat. Hence, in order to remove the confounding introduced by fat intake, correlation coefficients between endpoints were calculated from the 10 animals within each fat intake group and then averaged across the four fat dietary groups.

Results

Average correlation coefficients, apart from the effects of dietary fat, among pairs of the measured effects at four weeks are listed in Table 1. There was a strong positive correlation between triglycerides and non-HDL cholesterol levels. There was a negative correlation between triglycerides and HDL cholesterol levels. That is, HDL cholesterol levels decreased as the triglyceride levels increased. All other correlations were relatively insignificant.

Table 1. Correlation coefficients at 4 weeks of dietary exposure to fat, PFOA, and PFOS.

Endpoints	5% fat	15% fat	25% fat	35% fat	Average
PFOA / PFOS	0.38	-0.32	-0.06	0.50	0.125
PFOA / Triglyc.[a]	-0.46	-0.75	0.18	0.56	-0.118
PFOA / HDL chol.[b]	0.38	-0.07	-0.19	-0.42	-0.075
PFOA / non-HDL	-0.37	-0.63	0.25	0.39	-0.090
PFOS / Triglyc.	–0.10	0.57	-0.51	0.44	0.100
PFOS / HDL chol.	0.03	0.45	0.52	-0.23	0.192
PFOS / non-HDL	-0.46	0.54	-0.31	0.36	0.032
Triglyc. / HDL	0.23	-0.05	-0.68	-0.73	-0.308*
Triglyc. / non-HDL	0.71	0.87	0.23	0.90	0.678**
HDL / non-HDL	0.56	-0.16	0.23	-0.65	-0.005

[a] Triglycerides

[b] Cholesterol

* $P<0.10$

** $P<0.01$

In addition, multiple regression analyses were employed in order to simultaneously examine the effects of dietary fat, PFOA, PFOS, triglycerides, plasma HDL cholesterol, and plasma non-HDL cholesterol. For example, for plasma triglycerides the following model was obtained:

$$\text{Triglycerides (mmol / L)} = -1.13 + 0.173\cdot(\%\text{ dietary fat}) - 0.0036\cdot(\%\text{dietary fat})^2 - 0.4915\cdot(\text{HDL mmol / L})^2 + 0.5253\cdot(\text{ non-HDL mmol / L}) - 0.0178\cdot(\text{non-HDL mmol / L})^2$$

In this study, triglyceride concentrations were associated with dietary fat, HDL and non-HDL cholesterol. Quadratic (squared) terms were considered for each endpoint in order to account for non-linear relationships. The relationship between triglycerides and HDL cholesterol was adequately described by the quadratic term without a linear term. Note the absence of PFOA and PFOS in the resulting regression model. The PFOA and PFOS plasma levels in the study did not have a significant effect on triglyceride concentrations.

Similarly, the multiple regression analysis indicated that HDL cholesterol was affected only by dietary fat:
HDL cholesterol (mmol / L) = 0.369 + 0.0284·(% dietary fat)

Also, non-HDL cholesterol was only associated with dietary fat:
non-HDL cholesterol (mmol /L) = 15.17 – 0.758·(% dietary fat) + 0.0122·(%dietary fat)^2

In conclusion, the levels of PFOA and PFOS in this study were not significantly associated with triglycerides, HDL cholesterol, or non-HDL cholesterol levels.